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FERMENTATION ORGANISMS

FERMENTATION ORGANISMS

A LABORATORY HANDBOOK

14326

BY

ALB. KLÖCKER

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AND REVISED BY THE AUTHOR

WITH 146 ILLUSTRATIONS IN THE TEXT

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IN

HONOUR AND GRATITUDE

TO

PROF. EMIL CHR. HANSEN, PH.D.,

BY

THE AUTHOR.

FROM

THE AUTHOR'S PREFACE

TO THE GERMAN EDITION

In the course of a number of years, during which I had the honour to act as assistant to Prof. Emil Chr. Hansen at the Carlsberg Laboratory, and to assist him in the practical courses of instruction conducted there from time to time, the wish was frequently expressed by students to have at their disposal a handbook which would contain an adequate description of the fittings, apparatus and methods of a fermentation laboratory, as well as of the biology of the organisms of fermentation; in short, a guide by means of which the usual experiments of such a laboratory can be carried out. A similar request reaching me from the well-known publisher, Max Waag, of Stuttgart, I could but conclude that a real necessity existed for such a book.

The contents of the present work are divided into three sections. The first of these contains a description of the manner in which the science of the organisms of fermentation has gradually developed; at the same time, an indication is given of the most important steps which have marked the progress of our science.

The second section describes the fitting up of the laboratory and all that is necessary for conducting work. Laboratory methods are then explained, special attention being given to the preparation of pure yeast cultures in large quantities. Finally, the third section treats of the most important micro-organisms of the alcoholic fermentation industry. The book thus deals with that domain in which Hansen has opened up so many new paths.

For each section there is a bibliography which embraces the most important researches, and contains explanatory notes. The literature after 1st January, 1900, could not be included. In certain cases I have made experiments for the sake of confirmation, and have quoted some results not hitherto published.

The branch of the fermentation industries with which my book is chiefly concerned is that of brewing, which was the first to make use of Hansen's pure culture system, and hence to adopt a rational mode of working. Brewing has thus, to a certain extent, become the model for the other branches of the alcoholic fermentation industry. The science of the organisms of fermentation as set forth in this book deals, however, not only with practical applications, but also with important theoretical aspects of chemistry and botany.

ALB. KLÖCKER.

COPENHAGEN, *January* 1900.

PREFACE TO THE ENGLISH TRANSLATION

BY PROFESSOR ADRIAN J. BROWN, M.Sc., F.I.C.

A VERY considerable and rapidly increasing amount of attention is now being given in this country to Technical Microbiology in its relation to the fermentation industries, and consequently there is a growing demand for sound textbooks on the study of the "fermentation organisms" for the use of students who are taking up this special branch of work. But it is continually being forced on the notice of the writer, whose work is intimately connected with the teaching of Microbiology, that this demand is very inadequately satisfied at present. Whilst we are almost too well supplied with textbooks on Bacteriology as related to the organisms of disease, the number of books in our own language dealing with the subject of "fermentation organisms" is very limited, and this is especially the case with works describing the more modern developments of experimental method connected with the study of these organisms. For this reason the publication of an English translation of Herr Klöcker's *Gärungsorganismen*, a work specially devoted to the treatment of laboratory methods employed in the study of "fermentation organisms," should be welcomed by all teachers and students of Technical Microbiology, for they have now placed in their hands a book which cannot fail to be of great assistance to them. The special merit of this book requires no recommendation here, for it is written by one who is a specialist in his subject, and whose name is well known, not only for his own valuable researches, but also as a distinguished assistant of the illustrious Dr. Emil C. Hansen, to whom every one connected with technical fermentation in this country and abroad is so deeply indebted. The translators of the work, Mr. J. H. Millar and Mr. G. E. Allan, have been most successful in the performance of their task, and we congratulate them on a volume which should be the laboratory companion of every student of Technical Microbiology in this country. We believe also that this book will be a useful addition to the library of the pathological bacteriologist. Pathological bacteriology, owing to its phenomenal growth, is inclined to forget its past history and lose all connection with the older branch of microbiology from which it originally sprang. We think it will be found that the development in method of experiment and research in connection with the study of "fermentation organisms" still deserves the careful attention of bacteriologists, and confidently recommend this book to their notice.

ADRIAN J. BROWN.

School of Malting and Brewing,
The University, Birmingham,
16*th October*, 1902.

THE translators desire to express their best thanks to Mr. T. H. Pope for his great assistance in reading the manuscript and proofs of this translation during its progress through the press.

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SECTION I.

INTRODUCTION.[1]

In this text book the author has endeavoured to give a review of the biology of fermentation organisms in relation to the use of these organisms in fermentation industries, and especially in the manufacture of beer. In spite of this limitation, however, the contents are of very varied character, and branch off in different directions. The book is not a text-book of the chemistry of fermentation or of technical fermentation in the ordinary sense of the term.

For the high degree of development to which our knowledge of the fermentation organisms has attained we are indebted to a large number of investigators whose work has been steadily progressing for many years. To understand the development of our science up to the present day, let us, in what follows, glance back along the path traversed, and note its turning points, each one of which has been productive of practical results, the value of which is recognised at the present day. The beginning was, of course, first made when the microscope came into use. This apparatus, so indispensable for the examination of fermentation organisms, was invented in the year 1590, but Leeuwenhoek, in Holland (1632-1723), was the first to employ it in making a close study of these forms of life. He was followed by a number of distinguished microscopists who all, more or less, added to our knowledge of the natural history of micro-organisms. All these were descriptive and systematic morphologists and not experimenters. Of the more distinguished microscopists who followed Leeuwenhoek we may mention the names of Otto Friedrich Müller (1730-85), in Denmark, and Ehrenberg (1795-1876), in Germany.

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[1] The bracketed numbers given in this section relate to the bibliography at the end of the book. To the latter we have appended explanatory notes bringing in many amplifications and explanations which could not find a place in this short introduction.

In the year 1822 Persoon gave to yeast the systematic name Mycoderma, a designation which seems to indicate that he regarded it as a fungus (mycoderma signifies fungoid film).

About the same time—in the middle of the thirties—Cagniard Latour (V. 1, 2), Schwann (VI. 1, 2) and Kützing (VII.) stated expressly that yeast is a plant. Meyen agreed with this view and gave to the new genus the systematic name of Saccharomyces (*i.e.*, sugar fungus) which it has since retained.

Considering the state of knowledge at that time, very valuable contributions to the natural history of yeast fungus were made by Eilhard Mitscherlich. It is evident from a paper published by him in 1841 (IX. 1), that this investigator recognised the substance invertin. In 1843 (IX. 2) he read a paper on the multiplication of yeast; he had observed under the microscope the phenomenon of budding, and had followed the development from a single cell.

Schwann, Cagniard Latour and Kützing expressed the opinion that it is the living yeast cell which excites alcoholic fermentation. In direct opposition to this vitalistic theory, Justus v. Liebig (1839-40) came forward with his theory of mechanical decomposition (VIII. 1). According to Liebig, every fermentation consists of molecular motion which is transmitted from a substance in a state of chemical motion, that is, of decomposition, to other substances the elements of which are loosely bound together. In his last work on fermentation (VIII. 2), he sought to bring this theory into agreement with the observations of Louis Pasteur on auto-fermentation. Liebig's explanation of the latter is that the cells contain a decomposing substance which produces sugar for the auto-fermentation. Although he at first looked upon yeast as a lifeless mass, an albuminoid compound, yet he came gradually to the view that it consists of living cells. But, in his opinion, there could be no question of fermentation being a physiological process: in this respect he held to his chemical conception.

At that time a vigorous dispute was taking place between the followers and the opponents of the doctrine of generatio æquivoca, *i.e.*, of spontaneous generation. Let us look somewhat closer at this doctrine. By spontaneous generation we understand the development of organisms from lifeless material without eggs, seeds or embryos. Needham (1745), an energetic supporter of this doctrine, was the first to make experiments endeavouring to prove it. For this purpose he heated meat extract in closed flasks, and, on organisms appearing in the flasks, he assumed that they had been produced by spontaneous generation.

Spallanzani (1765) showed, however (I.), that certain errors were made in these experiments; he sealed his flasks hermetically and boiled them for an hour, after which treatment no development of micro-organisms could be observed. From his experiments he concluded that the "eggs" of the micro-organisms are present in the air and only develop after they have found their way into the liquid.

On these experiments the foundation of the technique of sterilisation was laid, and a substantial addition made to the methods of cultivation.

The Swedish chemist and apothecary Scheele put Spallanzani's experiments to practical use in the sterilisation of vinegar by heating (II.). Appert, in France (1810), went a step further and used the method for preserving soup, beer, wine, etc. (III.).

In 1836-37 Franz Schulze (IV.) and Theodor Schwann (VI. 1) published the results of researches in which they sought to prove that air, when freed from its germs, that is, rendered sterile, can come into contact with a sterilised nutritive liquid without micro-organisms developing in the latter. The experiments of the last-named investigators were made in the following way: flasks containing nutritive liquid were closed with plugs fitted with bent glass tubes through which sterilised air was sucked. In order to free the air from its germs, Schulze passed it through sulphuric acid whilst Schwann subjected it to a high temperature. Their opponents, however, would not accept such proofs, but asserted that, in these experiments, the air had been violently treated, and, as a consequence, had suffered such a change that the inert matter could no longer be vitalised by contact with it.

Then in 1854 H. Schroeder and Th. v. Dusch (X.) showed that air can be freed from germs by filtration through cotton wool; thus the above-mentioned contention was disposed of. In fact, this method is still employed when we wish to sterilise air.

Belief in generatio æquivoca, however, did not yet die out. Not until 1860 was the victory won by Pasteur (XI. 4), who exposed all the failings of the experiments made by his opponents to prove the existence of spontaneous generation; in every case without exception he could prove that either an omission or an error had been made. In consequence of these brilliant researches the theory of generatio æquivoca fell more and more into ill repute. Up to the present time no single case of spontaneous generation has been experimentally proved.

It has been remarked above that the principles of sterilisation and also a substantial part of our culture methods are the result of the experiments made in relation to the doctrine of generatio æquivoca. For the recent development in this direction we are indebted chiefly to Pasteur and his school. The appearance of Pasteur marks a very great and important epoch.

Besides the above researches relating to the doctrine of spontaneous generation, we might also refer to another of Pasteur's important researches which interests us here, *viz.*, his investigation on lactic acid bacteria (1857). He describes lactic acid fermentation and finds microbes, which, as he assumes, cause this fermentation in milk (XI. 1); later on he mentions the same fermentation in beer and worts. He further proved (1861) that butyric acid fermentation is brought about by a special micro-organism (XI. 5). In addition we might mention his researches on acetic acid fermentation (1864 and 1868). Kützing had shown in 1837 that this fermentation is caused by a bacterium (VII.); but important progress in this direction was first made when Pasteur published his experimental studies on the subject (XI. 7).

Pasteur also made (1861) the discovery that certain micro-organisms thrive in the absence of free oxygen (XI. 5). He calls such forms anaërobic to distinguish them from those organisms to which free oxygen is necessary and which he terms aërobic.

In 1807 Chaptal had announced that the formation of a film on the surface of wine always precedes the souring of the wine; and, as already stated, Kützing had described the acetic acid bacteria (VII.), which form acetic acid in beer and wine; but Pasteur was the first to spread the idea that bacteria excite diseases in fermented liquids (XI. 8).

The method of preservation invented by Scheele and Appert is, thanks to Pasteur's works, becoming of increasing practical value. His name has also been connected with it, as the method is known as "Pasteurisation".

Pasteur's doctrine that bacteria are responsible for the diseases of fermented liquids gave rise to a demand for the use of pure yeast. Pasteur communicated (XI. 8) a process for the purification of brewer's yeast, recommending that it should be cultivated either in sugar solution with the addition of tartaric acid or in wort containing a small quantity of carbolic acid. He seeks thus to gain the desired end by chemical means. (In most cases indeed this process is successful as a means of purifying the yeast from bacteria.) It was then unknown that some of the most dangerous diseases of fermented liquids are brought about by foreign, "wild" yeasts, and that this process favours these particular forms at the cost of the good yeast, as was shown later by Emil Chr. Hansen (XIX. 2, 3, 6). In fact the Pasteur process led in a direction exactly opposite to that in which the desired end lay.

A practical consequence of the doctrine of bacterial diseases was the construction of apparatus by means of which it was attempted to keep the living germs present in air away from the brewery worts, and so to ward off any bacterial infection which might be contracted in this way. For this purpose Velten, a co-worker of Pasteur's, constructed closed cooling apparatus for the aëration and cooling of worts. That this apparatus did not then come into general use was a simple consequence of the fact that, as mentioned above, the yeast which was added to the carefully cooled worts was not pure in the sense in which we, in the light of Hansen's work, now use this expression. The replacement of the old cooling vessel by the new apparatus could only be of real use when a yeast was obtained which could be depended on.

Pasteur had (XI. 2, 3) from the beginning of his career combated the mechanical decomposition theory of Liebig. His investigations had led him, like Schwann, to the result that alcoholic fermentation only begins when yeast cells are present and that it is not possible to bring about fermentation by the mere use of a constituent of the yeast or even by means of chemical agents. "Fermentation," said Pasteur, "is life without air," and he believed that it is the want of oxygen that makes the yeast cells exciters of fermentation, these then taking oxygen from the sugar and thereby producing the peculiar decomposition. Pasteur's theory has not, as we shall see later, stood the test of time.

Nägeli (1879) in the main supports Liebig (XVIII.). He expresses his molecular-physical theory in the following words: "Fermentation is the transference of the conditions of motion of the molecules, atomic groups and atoms of the various compounds constituting the living plasma, to the fermenting material, in consequence of which, equilibrium in the molecules of the latter is destroyed, the result being their disintegration".

Traube's enzyme theory (1858) may be referred to here (XII.). According to this theory fermentation is explained as an effect due to the various enzymes contained in yeast and not to the yeast cell itself. This theory has lately been confirmed by the discoveries made by Emil Fischer and Ed. Buchner in the chemistry of fermentation.

E. Fischer's investigations (XXIII. 1, 2) on enzymes have not only brought to light new and important facts, but have also pointed to quite new views as to the nature of the processes concerned, and Ed. Buchner (XXV.) by submitting yeast cells to high pressure, succeeded in obtaining an extract capable of producing fermentation in solutions containing sugar. Thus the actual processes of fermentation are now, like enzyme action, included in the domain of organic chemistry.

Pasteur closed his studies in fermentation with his book, *Études sur la Bière* (1876), and proceeded to other fields of investigation where, as is well known, he gained still greater renown.

A few years previously (1870) the descriptive botanist and microscopist, Max Reess, had carried out a research (XIV.), which, considering the then state of the science, must be regarded as of importance. The spore formation discovered (VI. 2) by Schwann (1839), and observed later (1868) by Jules de Seynes (XIII.) in some of the fungi of alcoholic fermentation, was found by him to occur in many different species. He regarded this as the most important distinguishing characteristic of the genus Saccharomyces. Later investigations have confirmed the correctness of this view. On the other hand his statements of the conditions of this spore formation must be regarded as erroneous. He distinguished the species according to the appearance of the cells. He did not recognise the pure culture and could not therefore deal experimentally with the question of species. He used the form of the cell as the distinguishing character of the species, calling the ellipsoidal cells "Sacch. ellipsoideus," the sausage-shaped, "Sacch. Pastorianus," etc. It was proved later by Hansen that one and the same species of yeast can occur in all these different forms, and that, consequently, the shape of the cell cannot be applied in this way. The Reess species have therefore not found acceptance in modern experimental science.

We have now reached that point where interest was lost in the theoretical as well as in the practical side of the question. The technologists felt themselves deceived by the expectations aroused by the above researches on yeast. The facts taught them by science did not hold good in practice, and often, indeed, their position became precarious. Large sums of money were lost in the breweries on account of accidents during fermentation, accidents the causes of which could not be explained, and against which precautions could not be taken. The yeast was spoken of as something mystical. The view of the science at that time (1884) was depicted in the following expression of Thausing's (XXII.): "Science has given us fine researches on fermentation organisms and on the nature of fermentation, but it has yielded almost nothing of direct value to the brewery ; now, as before, the process of fermentation, so far as practical application is concerned, is veiled by a mystic darkness. The investigations of Hansen on the culture of pure yeast entitle us to great hopes; if they do not lie we are near the attainment of an end the importance of which cannot be sufficiently valued. In the first place, however, we have to reckon with the state of affairs as they stand at present." Similar pronouncements had already been made by Holzner (XVII.) and Lintner (XX.).

Some years before this Hansen had published some of his investigations; but only now was attention directed to him. As botanist Hansen began and completed the reform which inaugurated the new era in the biology of the fungi of alcoholic fermentation, and also in fermentation technique as a consequence of the practical results achieved.

In 1880 and 1881, he conducted experiments on the micro-organisms occurring in air at various times of the year. During these researches he observed a characteristic which enabled him to decide whether a flask contains a pure culture of a yeast fungus or not, and on this he founded his first pure culture method. At the same time he began his experiments on the diseases produced in beer by yeast fungi, and expressed the belief that the wild yeast forms sometimes produce as great disturbances in fermentation industries as bacteria do. A little later he arrived at new points of view for the investigation of species, and indicated the outlines of spore analysis. The results of these pioneer investigations are to be found as short notes interspersed throughout his second treatise on the micro-organisms of the air (XIX. 2) which appeared at the beginning of 1882, but remained unnoticed at the time. His treatises published in 1883 formed, however, the real turning point (XIX. 3). At this time he had probed these questions to such a depth, that he was able to inaugurate a reform in theoretical as well as in practical relations.

In connection with pure culture methods, we have mentioned in the foregoing that Mitscherlich (IX. 2) had, in 1843, observed the budding of single yeast cells under the microscope. Of his successors Brefeld (1874) deserves particular mention as the one who brought to a high degree of perfection this method for studying the morphology and life history of different fungi (XV. 1, 2). But the procedure followed by these investigators did not suffice when absolutely pure cultures of micro-organisms were required in large quantities such as are necessary for physiological experiments; the requirements are then quite different, and accordingly the efforts of the subsequent investigators were specially aimed at working out a process to suit this case.

Here we must place in the front rank Lister (1878), who sought to prepare pure cultures of lactic acid bacteria by distributing them in the culture liquid (XVI.). He diluted down until only some of the culture flasks contained a growth, and from this he then infers that the flasks which show development each contain a pure culture.

But this method affords no security, and Hansen therefore, in 1880-81, worked out his first method, which has been referred to above. He made the important observation that the yeast cells, after they have been well shaken up in the flask containing nutrient liquid, sink to the bottom, and form there distinct and well-separated spots of yeast. Examination showed, as was to be expected, that those flasks, in which only a single yeast spot had developed, contained a pure culture. This observation was a considerable step forward. With this method Hansen combined cell-counting by means of a cover glass divided into squares. This rendered it possible to sow a single cell in each flask, and an exact method of preparing pure cultures in large quantities was thus obtained.

At the same time Robert Koch published his investigations on pathogenic bacteria, and, like Hansen, he felt the need of a satisfactory pure culture method for the preparation of mass cultures. Nutrient gelatine was brought by him into extensive use in bacteriology (XXI. 1). His first method (1881) for pure culture consisted in dilution in nutrient gelatine. Before the viscous gelatine had completely set, it was stroked with the point of an inoculation needle which had previously been in contact with the growth from which the required pure culture was to be prepared. The last streak made in this way may contain isolated colonies. The method was, as one can see, a very imperfect one, and Koch soon introduced another, *viz.*, that of plate cultures (1883) (XXI. 2). In this method the germs are distributed in liquefied gelatine, and are, by this means, more thoroughly dispersed. Like Hansen, Koch also observed the single spot; but the last-named method is not so sure as that of Hansen, in which the cells can be more uniformly distributed owing to the use of a liquid nutrient medium. In Koch's plate culture a colony very frequently showed itself to have been derived from more than one cell; the possibility consequently arises that several species may be mixed together in one colony.

Shortly after Koch had communicated his plate culture method, Hansen published his second pure culture method (XIX. 4, 5). The dilution, in this case, is carried out in nutrient gelatine, but the starting point is from the single cell which is controlled under the microscope. With respect to their accuracy, Hansen's two methods are equally good, the substitution of gelatine being made as it appreciably lightens the work connected with the preparation of the pure culture.

From the above it is plain that Koch's method does not conform with the requirements strictly necessary for a starting point, as does that of Hansen, since the latter starts from a single cell. With bacteria, however, this cannot be entirely carried out. Koch's method is much more suitable for separating the various elements of mixed cultures, so that one does not isolate only that kind which occurs most frequently, but also most of the others. His method also acquired the distinction of bringing nutrient gelatine into general use as a culture medium; from the appearance which the growths have on this medium, the characters of the species can to some extent be made out.

In course of time the number of species—belonging to various divisions of the fungus system—which were treated in Hansen's investigations on the organisms occurring in beer and beer worts became very large. His first treatise (XIX. 1) on fermentation organisms was published in 1879. Among bacteria, he made a special study of those of vinegar. He explains hitherto unknown differences in form, and shows how to recognise the conditions causing these variations.

What we know of the morphology of these species is due chiefly to him (XIX. 9). He further studied mould fungi and the fungi of alcoholic fermentation generally, but more especially the Mucors and the Saccharomycetes. His experimental researches on this subject are contributions to the general biology of the whole of the fungi (XIX. 8). As an example we might cite his researches on the circulation in nature, on the life cycle and on the conditions for spore and film formation. He gives definite methods for bringing into play the last-named functions, so that what was formerly entirely beyond control can now be brought about with certainty. There might be named, in addition, his researches on the germination of spores, on the relation between the form of the cell and the conditions of culture, on the limits of life of cells, using different methods of preservation, on the behaviour of species towards the sugars, etc. These investigations became likewise of immediate importance for the recognition of species ; new points of view were here brought to light, and this question was sifted to a depth not hitherto attained. The above-mentioned group of investigations showed that the Saccharomycetes, under certain methods of treatment, appear with constant characteristics, and that we can here, as with other fungi, make a separation into species, a point which was doubted by several investigators at the time when Hansen began his work. Another part of Hansen's work treats of variation (XIX. 8). He shows how, under certain conditions of culture, the characteristics can vary, that these variations are either temporary (variation of the cell form, variation of alcohol production) or permanent, and how the latter retain their characteristics through endless generations and under all methods of treatment (sporeless and filmless varieties). Theoretically these investigations are of special interest as showing that, in the apparent irregularity of the variations, conformability to law prevails. For the fermentation industries their importance lies in the fact that they show how new and permanent races can be prepared with certainty.

Having mentioned, in the foregoing, chiefly the theoretical works of Hansen, we will now give a review of his practical investigations. But, in fact, we can draw no sharp line, since here theory and practice constantly go hand in hand. Practical difficulties, with which the two Carlsberg breweries and also the Tuborg brewery in Copenhagen had to contend, induced Hansen, confident of success, to strive with all his energy to effect a fundamental reform. The practical consequence of his theoretical investigations was, on the one hand, the elimination of the disease yeasts; on the other, the separation of the culture yeast (the Saccharomyces cerevisiæ of former investigators) into several species and races, and, finally, systematic choice from the latter. This choice forms the most substantial part of Hansen's pure culture system, and was based on the study of these species from new points of view. Finally, he worked out a new method for the analysis of brewery yeast (XIX. 10). It was then made clear how very different these species and races are, and how each gives a special character to the liquids fermented by it. The great differences of the yeast showed themselves in a surprising manner, especially when the so-called wine yeast, Saccharomyces ellipsoideus, was separated into its systematic units.

Hansen published, in 1883, not only theoretical considerations, but, at the same time, the results of experiments which he had carried out in the old and new Carlsberg breweries in Copenhagen. His new system was worked out to the smallest details, and had also been tested in practice, so that it could be applied at once without any preliminary experimenting. As one may gather from the above, Hansen himself introduced his system for the bottom fermentation breweries. This system spread quickly into different countries and found an entrance not only into the breweries, but gradually also into the spirit and pressed yeast industries, and the manufacture of wine.

The relation between Pasteur's and Hansen's work in this respect was clearly and forcibly expressed by Delbrück in a lecture (XXIV.) delivered in Berlin in 1895: "Looking back on the last twenty-five years, there are two great epochs marking the scientific development of brewing; Pasteur's work, which was done after 1870, and which is adopted in principle when we nowadays strive, by the setting up of cooling vessels, to ward off external infection, forms one epoch; Hansen's, the other. But Pasteur's attempts could not lead to a fruitful issue, because one link was missing which was furnished by Hansen in his systematic choice of pure yeast. These two men and their discoveries have been the moving forces of the last decade, and have brought brewing to what it is to-day."

By Hansen's discoveries, the subject of micro-biology and fermentation technique here treated was given new life, and an impulse was imparted to the formation of a rich literature. From that time onwards, the interest of technical fermentation laboratories is claimed especially by the Saccharomycetes; and now new laboratories for promoting the industry are being erected in which biologists work side by side with chemists, where formerly the latter monopolised the whole field of work. An ever increasing body of distinguished investigators has taken up the subject, and their names and work will be given in the following sections, where the subject, as reviewed in this introduction, will now be examined more closely and treated in fuller detail.

SECTION II.

THE LABORATORY.[1]

MICRO-BIOLOGY has, during its work in the service of the alcoholic fermentation industries, developed a special technique and elaborated special methods; its research has assumed a character of its own, as may indeed be seen in the fitting up and in the apparatus of the laboratories which are now to be found in many places, sometimes as private laboratories, sometimes as state institutions. Some are purely for research, as, for example, that at Carlsberg, and their work is to promote the science of fermentation organisms by scientific investigation in a theoretical and practical direction; others, and the most belong to this class, are designed to serve practical men by furnishing them with analyses, and providing them with pure cultivations of selected species and varieties of yeast. Laboratories for the study of fermentation organisms are now also to be found attached to a number of the technical colleges (Hochschulen). These were set up for educational purposes after it had been recognised what an important influence the study has on the scientific instruction and on the industrial activity of the manufacturer.

As the number of laboratories increased, and according as they were constructed for the study of special branches of the fermentation industry, the outfit which had been formerly characteristic of these laboratories naturally experienced changes in regard to fittings, etc. The influence of the individual opinion of different managements also contributed to these changes. For the following description, which comprises only the biological part and not the chemical, we have taken as a pattern sometimes the Carlsberg laboratory, sometimes certain brewery laboratories fitted up for purely practical purposes, which the author has had an opportunity of inspecting; brewing is chosen by preference to exemplify the application of the methods in practice. In general this description applies also, as may be supposed, to those laboratories which are associated with other branches of the fermentation industry.

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[1] The bibliography will be found at the end of the book.

I.—FITTINGS AND APPARATUS.

1.—*General Principles for fitting up the Laboratory.*

In many cases, when a laboratory of the physiology of fermentation has to be fitted up, the site will have been fixed so that there is no choice; the best has therefore to be done with the space at one's disposal. If there is any choice in the matter, a site facing the north is to be preferred, as sunlight is not only very troublesome in microscopical work, but is also fatal to most micro-organisms. Further, it is also very much to be recommended in cases where there is sufficient room, that the space be divided in such a way that there is a small room to be used only for work with yeast and bacteria, and a larger one in which are placed the microscope table, cupboards for cultures and apparatus, a working bench, etc.; work on moulds, for example, might be performed on the last named. The conidia of moulds, being developed on the exposed surface of the nutrient medium, cannot be retained by the liquid, and, on account of their lightness, are very easily carried about by the air from place to place. Special precautions are therefore necessary when working with moulds; the best way is, as said before, not to work with moulds in the same room where yeast and bacteria are being studied.

It is therefore necessary to provide a place as free from dust and germs as possible. For dust always contains germs of micro-organisms—of bacteria as well as of moulds and yeasts. The surface of all fittings in the laboratory should therefore be as smooth as possible, without projecting corners or hollows in which dust can settle. There ought to be at hand no more apparatus than is absolutely necessary: cultures and apparatus should therefore usually be kept in cupboards: only those objects should stand on the tables which are being used at the time, and these should be put away again after use. Only in this way is it possible to keep everything dust-free and clean. The laboratory should appear as if nothing were being carried on even at the time when most is being done. If such a system is once introduced into the working of a laboratory time is economised and security is ensured during the progress of experiments.

Cupboards and drawers ought therefore to close tightly so that dust cannot force its way in; this is attained by providing the cupboard doors and the drawers with overlapping edges. A suitable height for the working table is 97 centimetres (about 38 in.).

Preparation of Bench Surfaces.—The working tables must be prepared so that they can stand washing with spirit, as many experiments have to be performed on a wet bench. This can be done in the following way: two solutions are prepared, (1) an almost saturated solution of aniline hydrochloride in water, and (2) a solution of 1 part of potassium chlorate and 1 part of copper sulphate in 120 parts of distilled water. Solution (1) is first rubbed into the wood and then solution (2), the solutions being used alternately until the wood has become sufficiently black. The one solution must have soaked into the wood before the other is applied. If the aniline salt partially crystallises, the bench must be moistened with warm water before solution (2) is applied. If it is found from the colour of the wood that one of the solutions has been used in excess, the other solution is applied twice successively. As a rule it is better not to use too much of solution (2), as in this event the wood becomes green instead of black. When this treatment of the bench is complete the wood is well rubbed for some time with linseed oil varnish. Repeated washings with lukewarm water will often be necessary, especially if the colour rubs off.

The above described preparation was much used formerly, but there are also other means for producing such a surface. From experiments made by the author the following recipe seems better than the first. Two solutions are also used in this method, *viz.:* (1) 600 grams of aniline hydrochloride are dissolved in 4 litres of water, and (2) 86 grams of cupric chloride, 67 grams of potassium chlorate, and 33 grams of ammonium chloride are dissolved in 1 litre of water. Immediately before use, 4 volumes of solution (1) are mixed with 1 volume of solution (2), and the wood is treated with this mixture once a day for four or five days. Afterwards the bench receives an application of the linseed oil varnish. The black colour develops more quickly than by the former process.

Solutions for Washing the Bench during Work.—The mixture of spirit to be used for washing the bench consists of 66 parts of boiled water and 33 parts of concentrated spirit. The sponge used in washing the table is kept in the mixture when not in use. In some laboratories an aqueous solution of mercuric chloride (1 gram per litre) is used instead of spirit.

The Microscope Table.—The microscope table is most conveniently situated when facing the north. The proper height of the table is about 86 centimetres (about 34 in.), that of the stool belonging to it 62 to 63 centimetres (about 24 in.).

The Sterile Room.—It is especially desirable, as stated above, to fit up a small room where only experiments with yeast and bacteria are performed. The Carlsberg laboratory has two such "sterile" rooms. The windows, which are double, are well sealed so that no draught can set the air of the room in motion. Where the windows do not look to the north, the panes are of frosted glass or are painted over. Curtains are absolutely to be avoided. The walls and the ceilings are painted with enamel which gives a perfectly smooth surface and also resists moisture; this is of importance when these surfaces have to be washed down or if special precautions have to be taken, as, *e.g.*, when the air in the room is to be made very damp in order to purify it, the germs floating in the air being then precipitated. This is accomplished by keeping the room full of water spray for some time by means of a small sprayer, after which the room is left quiet before it is used. The floor is covered with linoleum so that all cracks and clefts are covered. Pipe systems in the room are avoided; the gas pipe just passes through the wall and ends in a stopcock to which rubber tubing is attached leading to the Bunsen burner on the working table. In addition to the latter there is a small table furnished with drawers above and cupboard below containing spatula, forceps, inoculating needles (brass rods or pieces of platinum wire fused into glass rods), and also a selection of the nutrient liquids most used and empty sterile flasks.

In working with flasks a Bunsen burner is used which can be made luminous or non-luminous and gives a large or small flame.

To complete the equipment of the room, a shallow dish of tinned copper is required. This is used sometimes when working with a Pasteur flask, sometimes for holding the sterilised spatulas or inoculating needles which are set to cool here after sterilising in the flame, the dish being covered with a glass plate sterilised in the same manner.

2.—*Hansen's Sterile Cupboard.*

If the circumstances are such that a sterile room cannot be obtained, we must avail ourselves of the Hansen "sterile cupboard" and perform the finer kinds of work in it. This cupboard was the model for fitting up the sterile room, and is really a miniature of the latter. It is obvious that the cupboard ought also to be used when it is desired in particularly delicate work to take special precautions against infection.

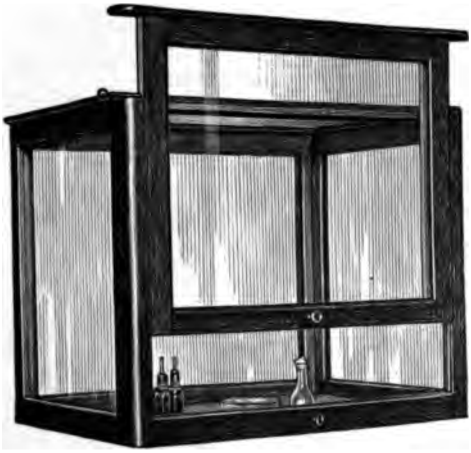

FIG. 1.—Hansen's Sterile Cupboard.

This cupboard (Fig. 1) consists chiefly of glass, only the framework and floor being mahogany. The latter is smooth, and is polished with linseed oil varnish, and bears washing with dilute spirit. The dimensions are about as follows: height, 56 centimetres (22 in.); length, 63 centimetres (25 in.); breadth, 50 centimetres (20 in.). The front side consists of a sliding door which can be kept open at any desired height. Before the cupboard is used it is washed inside and out, either with boiled water alone, with $\frac{1}{10}$ per cent. solution of mercuric chloride, or with dilute spirit; it is specially important first to brush and then to damp the surface where the door slides up and down, in order to prevent germs which settle in the groove from penetrating into the cupboard.

The cupboard is then closed and allowed to remain (usually for an hour), till the air inside has become quite still, and the water particles with which it is saturated have carried the germs present down tc the damp floor.

The cupboard must be kept sufficiently damp during experiments, as otherwise the germs are apt to be stirred up again.

In experiments where infected solutions or the like are liable to be spilt, it is an advantage to cover the floor of the cupboard with a zinc tray which can be easily removed and which can be cleaned and sterilised before and after use.

3.—*The Microscope and its Accessories.*

The microscope is one of the most important adjuncts in investigations connected with the physiology of fermentation. It will be shortly described here, and, in addition, references will be made to special literature where more detailed information may be obtained.

The compound microscope (Fig. 2) consists of two systems of glass lenses, the one nearer the object of investigation being called the objective, and the other nearer the eye, the eye-piece. All these lenses are fitted into a brass tube. The objective forms a real, enlarged and inverted image of the object which is being examined; this image is again magnified by the eye-piece. Thus the image that we see in a microscope is an inverted one. However good the lenses may be they never form exact images, and the error is increased by the eye-piece.

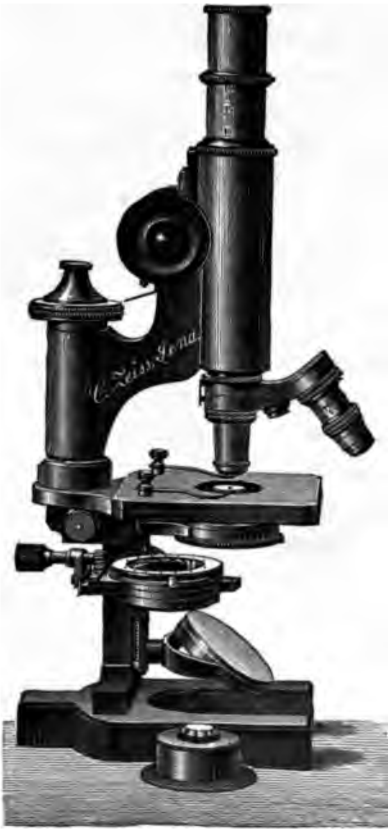

FIG. 2.—Microscope.

Spherical and Chromatic Aberration.—The errors caused by the objective arise mainly from spherical and chromatic aberration. Spherical aberration is to be ascribed to the fact that, of the rays of light which, diverging from a point, pass through a lens, the central ones are not focussed at the same point as the outer ones. The image is therefore hazy in outline. Further, white light, as is known, is composed of different coloured constituents, and on passing through the lens these are separated so that a coloured image is produced and the outline contains the familiar rainbow colours. To reduce spherical aberration various diaphragms are inserted in the tube which cut off the peripheral rays, and to eliminate chromatic aberration the lenses are composed of a biconvex and a plano-concave lens made respectively of crown and flint glass. This method gets rid of chromatic aberration almost entirely. In order to further correct for this and spherical aberration there is placed between the objective and the eye-piece a so-called collective lens.

Achromatic and Apochromatic Objectives. — Such objectives as we have described are styled achromatic; recently so-called apochromatic objectives have also been constructed. These are made of special kinds of glass (borate, phosphate, baryta and fluoride glass), by means of which more perfect colour correction is attained. They are far more expensive than the first named, which are quite good enough for the ordinary demands of fermentation work.

The Tube.—The tube is so arranged that it can be elongated and can thus increase the magnification; frequently it is provided with a scale of divisions by which the lengthening can be determined. The tube is supported on a brass stand which carries, among other things, two screws, one for coarse and one for fine adjustment.

Correction Objectives. — Objectives for high magnifications have in some cases an additional adjustment for the varying thickness of cover glasses. In these there is a ring on the objective provided with a scale, the numbers of which correspond with cover glass thicknesses expressed in tenths of a millimetre; the ring is turned until the proper mark coincides with a fixed index.

The Condenser.—A centrally perforated stage, on which the preparation to be examined is laid, is also fixed to the stand; under this stage there is a mirror which serves to project the rays of light through the aperture in the stage, through the preparation, and so along the tube to the eye, thus providing the necessary light for observation. The mirror is double, being plane on the one side and concave on the other; the concave side gives the strongest light and is therefore used for the higher magnifications. Of late years other condensers have been used, especially that designed by Abbe. This apparatus, which may be seen in Fig. 2, lying in front of the microscope, causes a much more intense light to pass through the microscope. The light is regulated by means of a diaphragm, for it may be so intense as to make the preparation indistinguishable. With increasing magnification more light is required. A very suitable form of diaphragm is that known as the iris diaphragm (brought forward in Fig. 2), which can be easily adjusted so as to allow more or less light to pass through. If the Abbe condenser is not used there are circular diaphragms with openings of different sizes which can be brought under the aperture of the stage. The condenser is especially advantageous in the investigation of stained preparations of bacteria.

Immersion Objectives.—For very high magnifications, and in order to get specially good definition, immersion objectives are used. In these the objective lenses are very powerful (and therefore very small since their curvature is great), and are immersed in a drop of liquid (water or oil) which lies on the cover glass. Water immersion was introduced by Amici, whilst homogeneous or oil immersion was first suggested by Stephenson.

In order to understand the advantage of the immersion system over the dry it is necessary to know what the angular aperture and the numerical aperture of the lens are. The angular aperture is the greatest angle formed by two lines drawn from the focus to the edge of the lens. The numerical aperture is the product of the refractive index of the medium between cover glass and objective and the sine of half the angular aperture (Fig. 3). The greater the numerical aperture the more rays pass from the object through the objective. In the dry system the numerical aperture is always less than 1, for the refractive index of air is equal to 1, and half the angle of aperture must, of course, be always smaller than 90°, and its sine

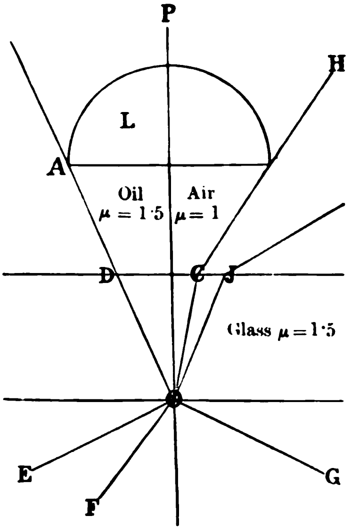

FIG. 3.—Diagram of a section through the front lens of the objective and the cover glass, showing the direction of the different rays of light with and without immersion liquid.

consequently less than 1. The refractive index of water is 1·33, but in practice the aperture for water immersion is never greater than 1·25; for cedar-wood oil and glass the index is the same, *viz.*, 1·52, but the aperture is not more than 1·40. The difference between the immersion and dry systems may be understood from the accompanying sketch (Fig. 3). This gives a section through the front lens, L, of the objective, the cover glass, and the intervening medium, that to the right through air (refractive index $\mu = 1$), that to the left through oil ($\mu = 1·5$). Two rays, EO and GO, proceeding from the object are refracted in passing through the cover glass towards the normal OP; GO in the direction D, EO in the direction J. When the ray, GOD, leaves the cover glass it passes into the immersion liquid, in this case oil, which has the same refractive index as the cover glass; the ray therefore will continue through the oil in the same direction as through the glass and will pass into the objective at A. If, on the other hand, the dry system is used instead of the immersion there will be air between the cover glass and objective with refractive index $\mu = 1$, so that a ray, EO, which leaves the cover glass at J does not pass into the objective at all. Only those rays which are less oblique than some ray such as FO will be able to strike the objective. It is thus seen that far more rays take part in the production of an image when the immersion system is used, and in consequence of this a much better image is obtained.

The strength of the oil immersion lenses is usually indicated by a fraction, *e.g.*, $\frac{1}{12}$, $\frac{1}{18}$, etc. By this is meant the equivalent focal distance of the respective lenses, expressed in inches.

Immersion lenses ought to be cleaned immediately after use by means of an absolutely dust-free linen rag which should be kept in a tightly closed box. A quite dust-free material should also be used for drying the lenses, for dust often contains particles which are capable of scratching glass. However, the lenses themselves should be moved and rubbed as little as possible. The liquid can be removed from the edge of the glass, when necessary, by means of blotting paper, and under certain circumstances the glass may also be cleaned with benzene or alcohol. The other parts of the microscope can be dusted by means of a soft brush.

The Stage is fitted up in various ways, *e.g.*, so that it is capable of rotation and of adjustment in different positions. These are, however, details, the description of which may be omitted.

There is sometimes a scale and vernier on the stage, the vernier being an arrangement which allows a finer reading of the scale to be made. It is a smaller scale running parallel with the fixed scale, and ten of its divisions are exactly equal to nine divisions of the latter. That division of the vernier which coincides with a division of the stage scale gives the required fraction in tenths.

Changing the Objectives.—There are various ways of attaching the objective to the tube. The objectives, which in some cases are simply screwed into the tube, may also be adjusted by means of a revolving arrangement (nose-piece), allowing several objectives to be attached to the tube at the same time; by simple rotation any objective may be brought into the required position. The objectives can also be changed by a sliding arrangement or with the aid of
These different arrangements enable one to change
s more easily and quickly.

eral rules for the testing of a microscope will
hat follows.

the Microscope.—There are usually given with
veral test objects; most frequently these

consist of scales from the wings of a butterfly (*Epinephele janira*) and a diatom (*Pleurosigma angulatum*). The microscope may then be tested by examining these under different magnifications. The latter are to be found in the table always supplied with the microscope. The first preparation is examined with a low magnifying power (60 to 150 times); the transverse marks on the scales ought then to be quite distinct. The silicious shell of the diatom has fine crossed markings; with a magnification of 400 to 500 these ought to be plainly visible; with a magnification of 150 to 200 and using oblique light they ought to be distinguishable. To obtain oblique light the concave mirror is turned so that the light enters from one side. With very strong immersion lenses the markings are resolved into a mass of small six-sided figures. In such a test the sharpness of the outlines ought also to be noted. Lastly, it should be ascertained whether the screws, etc., fit well.

When the microscope is not in use, it must be covered up so as to protect it from dust. A bell jar is to be recommended for this purpose, that half of it turned towards the light being painted with oil colour to protect the microscope at the same time from sunlight which, in course of time, affects the fine lenses, the mirror and the stand.

Slips and Cover Glasses.—In the microscopical investigation of micro-organisms glass slips and cover glasses are used. The growth is placed on the slip in a liquid and the cover glass laid on the top. The slips are rectangular, and in general 7·5 cm. long, 2·5 cm. broad, and 1·5 mm. thick. Cover glasses are of various thicknesses, thin ones being used for finer work. The use of thick glasses, *e.g.*, 0·20 mm. thick, is to be recommended for ordinary work. If mixed cover glasses are bought, they ought to be sorted according to their thickness, which can be determined by means of the apparatus described on page 34. The cover glasses used most are 18 mm. square; but round and rectangular ones of various sizes are also employed.

For several kinds of work cover glasses should be used which are etched with squares, the squares in some cases being numbered. Fig. 4 represents a squared cover glass, used by Hansen in his first pure culture method. The squares were used for determining the total number of cells which were present in a drop of any liquid. This drop was contained within the boundaries of the whole square. Hence the small size of the latter.

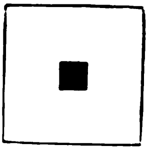

Fig. 4.—Hansen's Squared Cover Glass.

The most frequently employed squared cover glasses have larger squares (see Fig. 5 and Fig. 6). Will only numbers the squares in the top row and the left-hand column, but Alfr. Jörgensen numbers all the squares (Fig. 6).

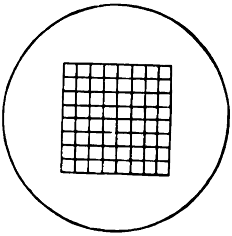

Fig. 5.—Squared Cover Glass much used.

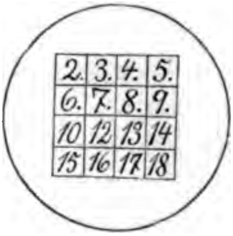

Fig. 6.—Jörgensen's Squared Cover Glass.

Since such cover glasses are somewhat expensive and can be easily etched, we describe a method given by Will for this purpose. A little wax is melted in a saucer and the cover glass dipped into it, being held at one corner by pointed forceps; it is taken out quickly and as much as possible of the melted wax allowed to run off, leaving on either side a thin even layer of wax, which is allowed to solidify. By aid of a very fine needle and a small ruler the required lines are then scratched on the wax and the cover glass immersed for a moment in hydrofluoric acid. If there is no silver or platinum crucible obtainable for holding the acid, a common porcelain crucible or dish or a watch glass can be used after being coated with paraffin or beeswax. After taking the cover glass out of the acid it is washed with water and then laid in warm water to melt off the wax; it is afterwards dried and placed in chloroform in order to remove any traces of grease. It is convenient to have a stock of these squared cover glasses, which are used in investigating the life history of development and for preparing pure cultures, as will be described later on.

The Micrometer.—It will be necessary in many cases to be able to measure those objects which we examine in the microscope. A micrometer is used for this purpose. It is usually in the form of a thin glass plate situated in the eye-piece and on which a certain number of equal divisions are etched. When the micrometer is in the eye-piece the size of the object can be measured by finding how many micrometer divisions the object covers. On the table of magnifications supplied with the microscope are usually to be found those numbers with which direct readings must be multiplied in order to get the true length of the object; the length is given in micro-millimetres, *i.e.*, $\frac{1}{1000}$ of a millimetre, a magnitude denoted by μ. This factor can be determined independently by using a stage micrometer, that is, a glass strip on which 21 divisions are etched, their distance apart being $\frac{1}{100}$ of a millimetre or 10 μ; with this micrometer on the stage and the other in the eye-piece it may be then noted how many divisions of the micrometer are equal to a certain number on the stage micrometer. If, for example, 2 divisions of the stage micrometer ($= 20\ \mu$) correspond with 7 divisions of the micrometer of the eye-piece, the apparent size expressed in divisions of the eye-piece micrometer have to be multiplied by $\frac{20}{7} = 2{\cdot}857$ in order to get the true length expressed in μ. The higher the magnification, the smaller is this factor.

Counting Apparatus.—In order to be able to count yeast cells a "net" eye-piece is used, consisting of a circular piece of glass on which is etched a square, divided up into sixteen or twenty-five smaller squares; it is fitted up in the same way as the micrometer in the eye-piece and is used in conjunction with a hæmatimeter. This (Fig. 7) is a glass slip (a), to which is fastened a cover glass (b), from the middle of which a circular piece has been cut out. A drop of liquid containing the cells to be counted is placed in the shallow space thus formed and enclosed by an ordinary cover glass. The thickness of the perforated cover glass is usually 0·1 or 0·2 mm.

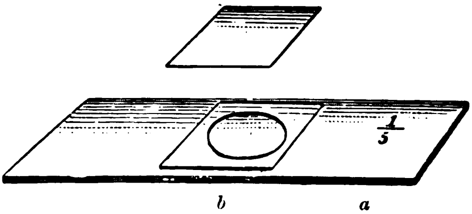

Fig. 7.—Hæmatimeter. (a) The object glass ; (b) the cut-out cover glass fastened on to it. (After Hayem-Nachet.)

Thoma's hæmatimeter (Fig. 8) is also used for counting micro-organisms. A is a glass slip, on which a cover glass (a) is fastened which has a circular hole in the middle and is 0·2 mm. thick. A circular cover glass (c), 0·1 mm. thick, is fitted centrally in this hole and is also fastened to the glass slip; thus, an annular space (*d*) is formed. In the middle of (*c*), two sets of twenty-one parallel lines are etched which cut each other at right angles. There are thus formed a large square with a side of 1 mm., and small squares with a side of 0·05 mm. The drop of liquid to be examined is placed on this square and enclosed by the cover glass (*b*), the depth of the liquid layer (*e*) thus formed amounting to 0·1 mm. *B* gives a vertical section of the chamber.

The method of counting is described later.

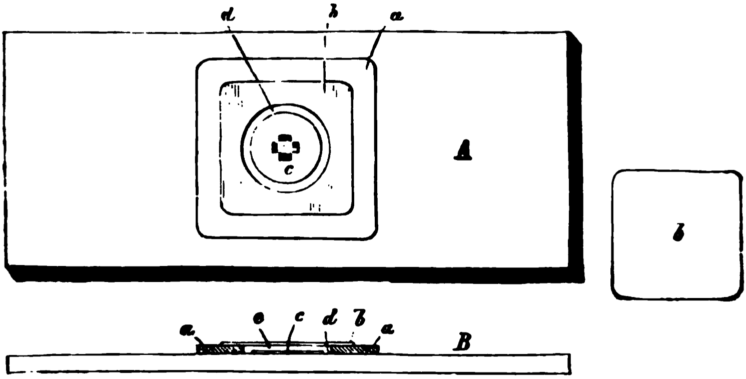

FIG. 8.—Thoma's Chamber. (*A*) View from above; (*B*) from the side; (*b*) a cover glass. (The significance of the remaining letters is given in the text.)

Klönne and Müller's Object Marker. — The object marker (Fig. 9) made by Klönne and Müller (Berlin) is often used in the course of the preparation of pure cultures and during observations on growth.

This instrument is used to mark a certain spot in a preparation by impressing a coloured ring round it on the cover glass. The apparatus is fitted on the microscope in place of the objective. There is an opening where the front lens of the objective would otherwise be. The lower half of the apparatus is capable of vertical movement, being fitted with a somewhat weak spring. The opening at the point must be quite flat and ought not to have a greater diameter than 0·75 mm. The method of using it will be referred to later.

Cover-Glass Gauge.—In the above it has been mentioned that objectives are sometimes provided with a correction so as to be adjustable for any thickness of cover glass. It is occasionally important to be able to measure the thickness of cover glasses. The instrument represented in Fig. 10 is used for this purpose. When the upper part is screwed down as far as possible, the zero of the scale on the screw-head coincides with a mark on the stem. In measuring the thickness of a cover glass the screw is screwed back, the cover glass inserted edgeways in the space thus made, and the screw again brought down until it touches the cover glass. The number which is now at the mark gives the thickness in $\frac{1}{100}$ mm.

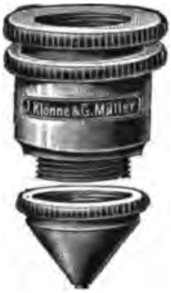

FIG. 9.—Klönne and Müller's Object Marker.

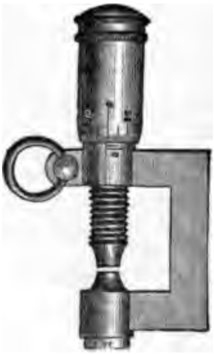

FIG. 10.—Cover-glass Gauge.

Apparatus for Artificial Illumination.—In microscopical work one must often resort to artificial light and so lamp is necessary on the microscope table. Electric light has the advantage of coolness; otherwise a gas or oil lamp must be used. As the continued use of a strong, yellow light is injurious to the eyes, it is better to let the rays from the lamp pass through a blue liquid, by means of which an agreeable greenish or bluish light is obtained which does not affect the eyes. Such a liquid may be prepared by adding ammonia to an aqueous solution of copper sulphate contained in a glass globe. The best illumination is then obtained by a suitable adjustment of mirror, globe and lamp. The positions of the microscope, globe and lamp can then be marked on the table so that these can easily be put into place at any time.

In addition it is as well to fix a screen between the microscope and the lamp, partly to protect the eyes from the direct light of the lamp, and partly on account of the heat.

If a gas lamp is used as the source of light, a damp cloth should be hung on the screen to keep the air moist.

Small Auxiliary Apparatus.—In addition to what has been described, various small articles are required, *e.g.*, glass spatula, platinum wire, forceps, preparation needles, etc. Two glasses may also be kept on the table for holding dilute sulphuric acid; in one, soiled glass slips are put, in the other, cover glasses; in this way the micro-organisms on the glass are killed and thus prevented from passing into the air after the glass has dried and before it is cleaned again.

Bottles for Reagents and Immersion Oil.—For microscopical work certain chemical reagents are indispensable. These are preferably kept in bottles provided with glass stoppers which are drawn out into the form of a rod; the latter reaches almost to the bottom of the bottle and in some cases has a thickened end. Figs. 11 and 12 represent two of the commonest forms of these bottles.

Fig. 13 represents a bottle which is made by Leybold's successors (Cologne) according to the design of Arthur

Meyer, and which has found general acceptance. It has this advantage, that the rim, which is enclosed in a cap, cannot be easily soiled since this is bent inwards into a funnel shape which compels the operator to draw the glass rod out of the middle of the bottle, the rim automatically removing superfluous liquid. The liquid which is removed at the funnel runs back again into the bottle; the latter should be kept quarter full, as, if upset, the rim prevents the liquid from running into the cap.

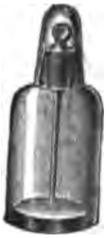

FIG. 11.—Bottle for micro-chemical reagents or immersion liquid.

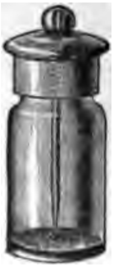

FIG. 12.—Bottle for micro-chemical reagents or immersion liquid.

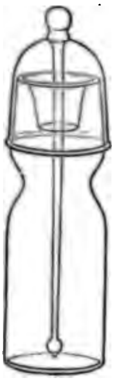

FIG. 13.—Arthur Meyer's bottle for micro-chemical reagents or immersion liquid.

4.—*Thermostats and their Accessories.*

Certain constant temperatures are necessary in fermentation work; such apparatus as will accomplish this and in which cultures can be placed are called thermostats or incubators. There are now numerous designs of these; we shall only mention a few here which have been found suitable. One of these, for instance, is the thermostat made by Rohrbeck (Berlin) which is depicted in Fig. 14.

Rohrbeck's Thermostat.—This consists of a double-walled copper box oval in section, covered with felt on top and on the sides. It is divided into two parts, the larger above, the smaller below; each section is provided with

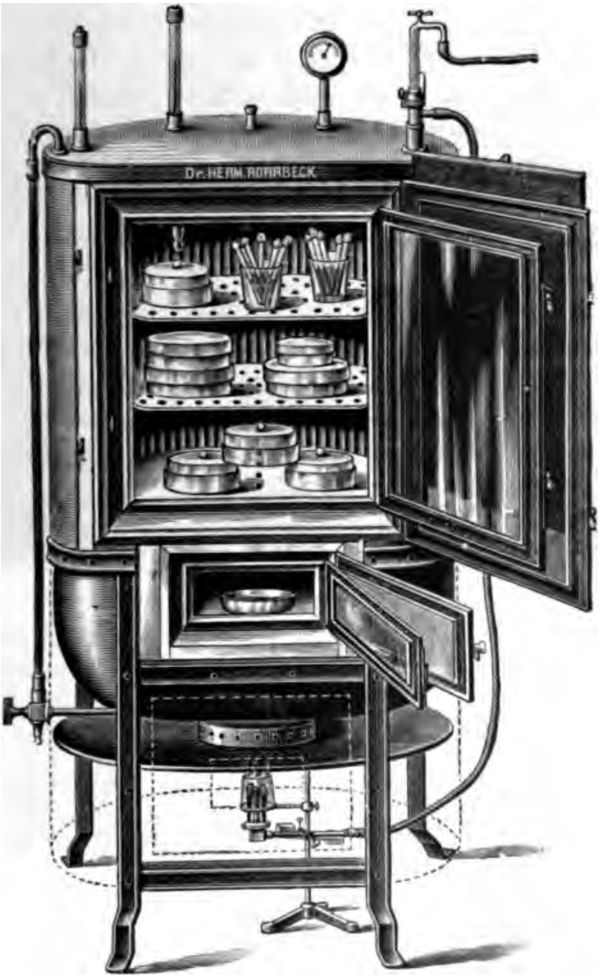

FIG. 14.---Rohrbeck's Thermostat.

double doors. The lower chamber is only used when moist air is required in the thermostat, a saucer of water being placed inside. The doors are made of glass, the outer one being covered with a piece of felt which can be removed.

The space between the walls of the box is filled with water; distilled water being used to prevent furring. The thermostat is provided at one side with a water gauge carrying a two-way cock, which allows the water either to enter the gauge or to be drawn off. In the top is an opening in connection with the space between the double walls of the box; this opening is used for filling the space with water, and, after this has been done, for holding a thermometer which passes through a cork and has its bulb dipping into the water. On the opposite side (to the right in the sketch) there is a similar opening to which a thermoregulator is fitted (see p. 46). There is a third opening, which is connected with the inside of the thermostat; a thermometer is placed in this by which the temperature of the chamber containing the cultures may be read. There are two other openings also leading to this chamber; they act as valves and are provided with caps which can be closed either completely or partially. Lastly there is an opening to which a hygrometer may be fitted. In the thermostat there are several movable shelves, which are perforated to allow of free circulation of air. Thermometers are required here also.

The heating is done by means of gas flames which are placed under the apparatus, as may be seen from the figure. The water is thus warmed, but in order to keep the temperature constant the thermoregulator is indispensable.

Quite a large quantity of water is used in such a thermostat, about 43 litres being required for a thermostat 40 cm. high, 50 cm. broad, and 25 cm. deep.

Two gas flames are used so as to reach the desired temperature quickly, but this process may be made still shorter if the thermostat is filled with water previously heated. The regulator is adjusted by being warmed in a water bath of the desired temperature before it is fitted to the thermostat.

Besides the above form there are numerous others. Muencke (Berlin) and Altmann (Berlin) also make good thermostats.

Panum's Thermostat.—In many investigations it is important to have at the same time a large number of chambers at different temperatures. Panum's thermostat (see Fig. 15), which has a series of constant temperatures, realises the required condition. We will describe in what follows its construction along with the improvements which this apparatus has undergone in the Carlsberg laboratory.

This thermostat consists of three cupboards soldered together which are designated in the accompanying Fig. 15 with the letters A, B-C, and D. The reason that it is not made of one cupboard divided into three parts is that A and D require more repairing than the middle one B-C. The former have therefore to be made separate from the latter. The inner dimensions of each of the compartments A, B-C, and D is about 63 cm. in length, breadth and depth. The first compartment, A, is double-walled, and is made of tinned copper; the jacket, *c*, is filled with distilled water, which is kept at the required temperature by a gas lamp, *a*; the gas flame is controlled by means of a regulator, *b*, which dips into the water through a tubular opening. This gas lamp is placed under a projecting wing, *d'*, of the external copper case; this wing is also tinned and in addition covered with asbestos paper, excepting the part immediately above the lamp which is thickened by the addition of another copper plate. As the wing is burnt through in course of time it is not put into permanent connection with the outer copper case but joined to it by screws and flanges with rubber packing so that it can be replaced when necessary.

There is also another tubular opening (not shown in the figure) at the top of the compartment A, and in front of

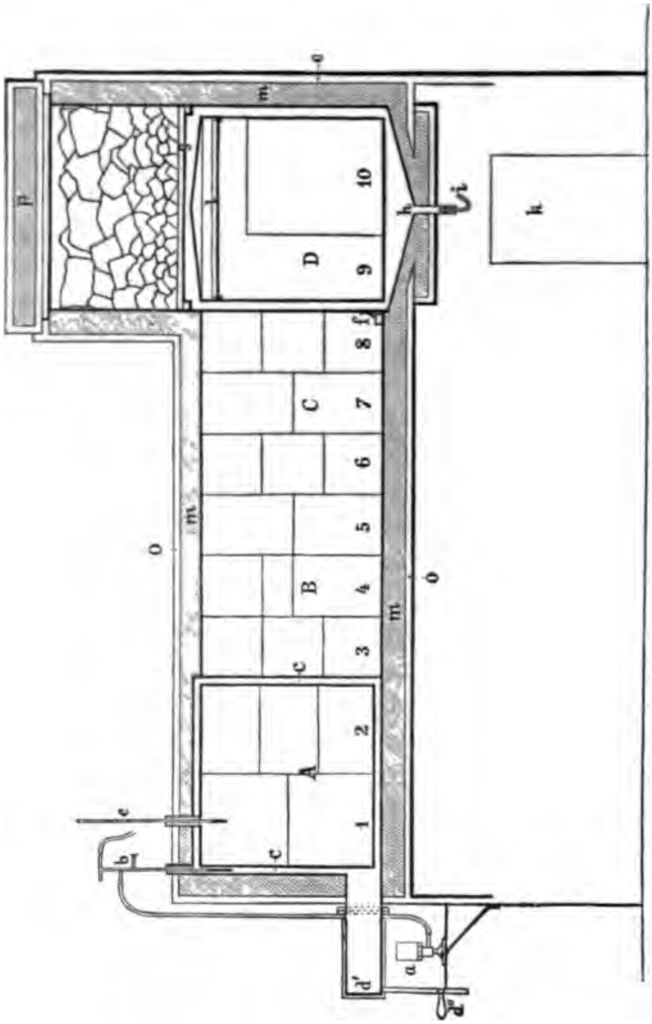

Fig. 15.—Section through Panum's Thermostat. (The significance of the letters is explained in the text.)

that holding the regulator, and through this a thermometer dips into the water to register its temperature. Water is run in through one of the above openings, and can be run off at the stopcock, *d''*, situated on the wing.

The space in the compartment, A, is divided into two parts, 1 and 2, by a partition; ledges, on which loose shelves can be laid, are fixed to the walls at various heights. There is an opening in the roof for a thermometer, *e*, to give the temperature of the air in the space A.

The other two compartments, B-C and D, are made of tinned sheet iron, the compartments D and C8 being carefully painted all over with red lead to resist damp due to the cooling in D. B-C is divided into two compartments B and C; each of these being again divided into three spaces (3, 4, 5, and 6, 7, 8), the walls of which are also provided with ledges for fitting up several stages. These are all made of tinned sheet iron. In compartment C8 there is a bent metal strip, *f*, soldered along the bottom against the wall next to D so that the condensation water which forms on this wall can pass over the strip and run into a long narrow box placed below.

The last compartment, D, is an ice box consisting of an outer and inner receptacle, the latter of these being cooled by water trickling down over it from a mass of ice resting on a strong grating, *g*. To distribute the water the inner receptacle has its roof sloping to the sides and to the back. The water is run off through the opening, *h*; in this is a cork passing through which there is a tube, *i*, forming a water trap; the water is caught in a vessel, *k*, placed beneath. The cork must be taken out every day in order to remove dirt coming from the ice. There is a movable trap, *l*, fitted to the top of D9, in which condensation water collects and is removed.

The whole apparatus is completely surrounded by a layer of felt, *m*, 8 cm. thick, and enclosed in a tight wooden box, *o*.

The ice holder is closed by an iron lid, above which is a wooden lid, *p*, provided with an 8 cm. layer of felt. The lid can be opened and shut easily by means of a counterpoise weight hung over a pulley fastened to the wall of the room behind the thermostat.

Each of the spaces 1 to 8 is provided with a tightly fitting glass door, and doors of sheet iron are fitted on each of the four large compartments A, B, C, and D, which are closed tightly by pressing against rubber strips fitted on to the partitions. Four corresponding doors, also shutting tightly, are attached to the wooden case, their inner sides being coated with woollen pads. All these doors are hinged below, and, when opened and resting in a horizontal position on adjustable brackets, may be used as tables.

The space under the thermostat is used as a cupboard.

If the apparatus is working and the regulator set, for example, at 40° C., compartment 1 will be at this temperature while the temperature in 9 will be only a few degrees above 0°. In the intervening compartments the temperature varies between these two extremes. In individual compartments the temperature varies from wall to wall and also from top to bottom.

If the temperature of the room containing the thermostat is somewhat high, as may happen in summer, it will be sometimes difficult to reach a low enough temperature in the cold part of the thermostat. A small iron box (Fig. 15, 10) with an ice holder is then placed in D; the temperature of the whole thermostat is thus lowered and at the same time a specially low temperature compartment is obtained.

Schribaux's Thermostat. — A thermostat frequently used is that of Schribaux which is seen in Fig. 16. It consists of a wooden cupboard with a copper floor; the heated gases of the burner pass through brass tubes fixed to the walls. A special regulator, mentioned on page 49, is usually employed along with it. The temperature variations are greater in this thermostat than in those of Rohrbeck and Panum, as the large doors when opened allow a considerable cooling to take place.

Large Warm Chamber.—In more extensive experiments, in which many cultures are dealt with simultaneously at the same temperature, it would, of course, be difficult to find sufficient room for them in an ordinary thermostat. If circumstances allow, a small room can sometimes be made into a thermostat. It is fitted up according to the same principles as an ordinary thermostat, and is, in fact, the same on a larger scale. The Carlsberg laboratory

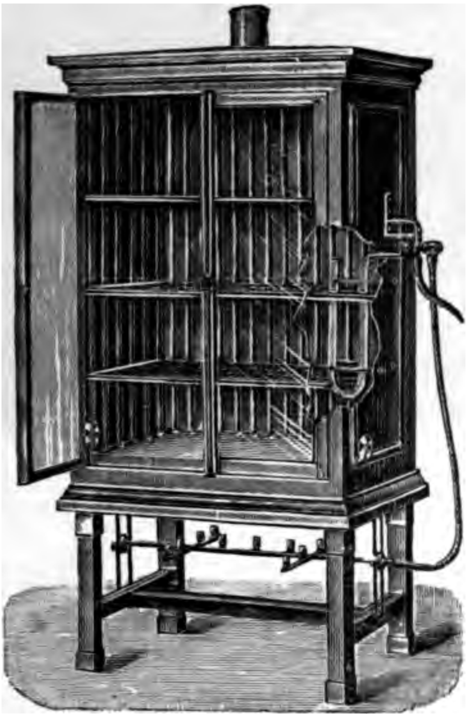

FIG. 16.—Schribaux's Thermostat.

contains a "warm chamber" of this kind, which is usually kept at 25° C., and which has proved eminently satisfactory. The room is 250 cm. high, 160 cm. long, and 160 cm. broad. Movable shelves are fitted along the walls. The walls and the roof are composed of two layers of hollow stones between which animal charcoal is placed; the door is also double and filled with kieselguhr. The heating is effected by means of warm water which passes from a vessel outside which is heated by a gas flame, circulates through copper pipings along the walls and so returns to the vessel. The piping is about 960 cm. long, 4 cm. in diameter, and is fixed about 55 cm. above the floor. A Reichert regulator is fixed in the wall and is in communication with the room; at another place a thermometer passes from the outside into the room so that the temperature of the latter may be observed from without. There are also two openings in the wall closed with cotton wool which act as ventilators. Through these, such things as tubing may be passed in.

A temperature of 15° C. is often necessary in the analysis of yeast. If a Panum thermostat is not available, a special thermostat for 15° C. must be fitted up. In many cases a cellar might be used instead of the above, although its temperature is of course not particularly constant.

A. Petersen's Thermostat for Low Temperatures.— The principle of a thermostat for low temperatures differs from that of the thermostats described where the temperature is above that of its surroundings; in this case the process is reversed. Anton Petersen has constructed such a thermostat for use in the laboratory of the old Carlsberg brewery in Copenhagen. The thermostat consists of a cylindrical double-walled copper box with a loose lid also double walled. The outside is covered with felt, and the space between the walls filled with water. Above on one side there is an inlet for the water, and on the opposite side an outlet. Near the inlet there are also perforations for regulator and thermometer. In the lid there are, in addition, two openings, one for a thermometer which projects into the interior of the space in which the cultures are placed, the other acting as ventilator. Tap water is used for obtaining the desired temperature, its temperature being generally lower than 15° C. The water passes from the tap through a tube to a small constant-level cistern, and from there through another tube to the regulator (see page 49).

Pfeiffer's Microscope Heating Apparatus.—When it is desired to follow out the development of a micro-organism at a certain constant temperature under the microscope, this can be done by using the small thermostat designed by L. Pfeiffer, which is shown in Fig. 17.

The arrangement consists of a mahogany box, which completely surrounds the stand, and which is almost airtight when closed. Its front wall has a glass window to admit sufficient light for observation; the left and right walls (seen from the observer) have each a well-fitting flap door so that the preparation may be manipulated. In order to make the microscope freely accessible, the side walls are capable of being completely removed along with the halves of the back wall, which is divided down the middle. The whole stands on a thick metal plate with three metal feet. The heating is effected by warming the plate from below by means of a micro-burner, the gas supply of which is controlled by a regulator. Experiments made in the Carlsberg laboratory have shown that the Reichert regulator described below is very suitable for this apparatus. With the side flaps opened or closed the greatest variation of temperature in the apparatus at 25° C. or 32° C. was only 1°. If the apparatus is closed and then the flaps suddenly opened, the temperature falls about

2° and then keeps constant. It is therefore desirable to leave the flaps open when working with temperatures which allow of this. With high temperatures this is impossible.

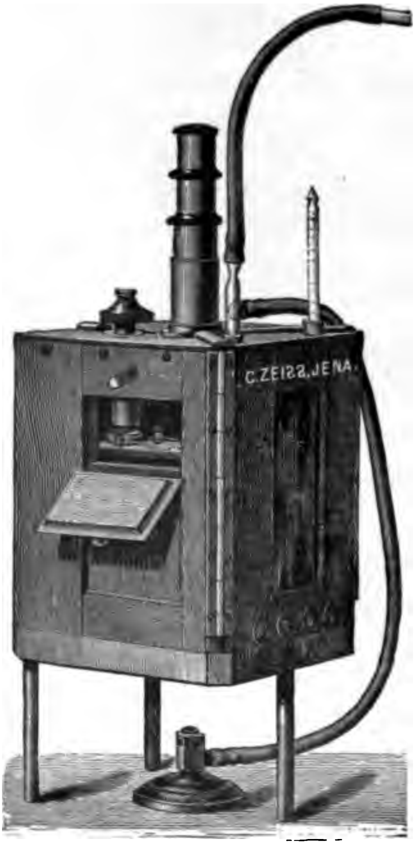
C. ZEISS, JENA.

FIG. 17.—L. Pfeiffer's Microscope-heating Apparatus.

Reichert's Regulator.—There are numerous forms of thermoregulators which may be used in combination with the thermostats for higher temperatures described above. In the course of long use in the Carlsberg laboratory and other places, Reichert's regulator has proved extremely efficient, this being due in no small degree to its simple construction. The principle of this regulator consists in the mercury expanding by heat and closing the inlet tube, thus diminishing the gas supply. The apparatus is represented in Fig. 18 at about one-fourth of its natural size; *c* is the bulb filled with mercury, the thermometer tube widening out above into a cylindrical space which communicates with the inflow tube, A, by a ground air-tight joint; the tube, A, reaches down to the point where the widening of the thermometer begins, and has a fine opening at *a*. The gas passes off to the burner through the side tube, B. In order to adjust for various temperatures, there is another side tube fitted to the thermometer stem and filled with mercury, its end being closed by an easily adjustable iron screw, S. The regulating takes place in the following way: The tube, A, is turned until the opening, *a*, is opposite the tube, B; the screw, S, is set so that the mercury just begins to fill the wide space when the proper temperature is reached; the mercury is then forced upwards by the screw until the flame begins to get smaller. As a result of the closing of the tube, A, the burner is fed only through the opening, *a*, the size of which can, in some forms of the regulator, be varied by turning the tube, A.

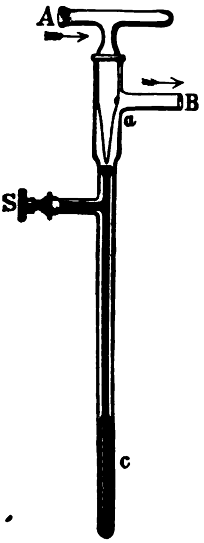

FIG. 18.—Reichert's Thermoregulator.

After being in use for some time, a black powdery substance is deposited on the surface of the mercury in consequence of impurities in the coal gas, the sensitiveness of the regulator being diminished. To remove this it is sufficient to take out the inflow tube for a moment and to remove the impurity from the mercury with a brush. The Reichert regulator allows the controlling of all temperatures from 1° above the surrounding temperature almost to the boiling point of mercury.

Fig. 19 represents the improved form of the Reichert regulator, the bulb being omitted. When the temperature becomes too high, the mercury raises the floating valve, *a*, *b*, and thus cuts off part of the gas supply. As in the simpler form there is a by-pass at *c*.

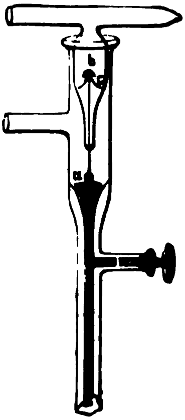

Fig. 19.—Reichert's Improved Thermoregulator.

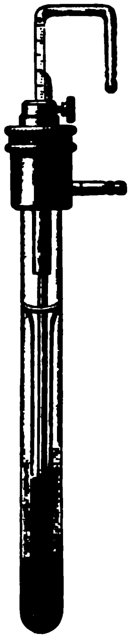

Fig. 20.—Muencke's Thermoregulator (after Lothar Meyer).

Muencke's Form of Lothar Meyer's Regulator. — Regulators are often employed in which there is, above the mercury, a liquid which boils at a low temperature, *e.g.*, alcohol or ether. This principle was first proposed by

Lothar Meyer, and has been applied in several ways. A regulator of this kind (by Muencke, Berlin) is shown in the illustration (Fig. 20).

The inflow gas tube is a metal one with a steel end; it passes through an air-tight stuffing box, and can be fixed by means of a screw. It is also provided with a millimetre scale so as to control its position.

This regulator is used in many laboratories, and is perhaps, under certain circumstances, to be preferred. But for most cases the Reichert regulator is quite sufficient.

Roux's Regulator.—Roux's regulator is the one usually employed with the Schribaux thermostat mentioned on page 42. This regulator consists of a steel and a zinc plate soldered together and bent in a U shape. One limb is fixed by a screw, the other remains free. When the temperature rises the two limbs separate, the free limb thus displacing a cone ventilator fitted into a metal box, so that the gas can only enter through a by-pass. If the temperature sinks, the free limb moves back and opens the valve, so that the gas passage becomes free.

Soxhlet's Regulator.—A different regulator from the above must be used for low temperatures. That of Soxhlet, which is shown in Fig. 21, is suitable for use with the Petersen thermostat at 15° C.

Cold water flows down from a constant level cistern through the upper tube on the right. If the temperature of the water in the thermostat is normal all the cold water runs off through the overflow vessel on the left, which is always full; but, should the temperature of the thermostat water rise 0·1° above the normal, the ascending mercury column closes the opening of the syphon, and the cold water flows through the horizontal tube on the right into the water of the thermostat, until the normal

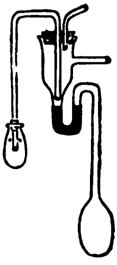

FIG. 21.—Soxhlet's Thermoregulator for low temperatures.

temperature is again reached and the mercury column has sunk so far as to leave the opening of the syphon free again. The bulb on the right contains a few drops of ether or some other liquid of low boiling point.

Koch's Lamp.—For heating the above described thermostats, gas lamps are sometimes used which are provided with an automatically acting apparatus that cuts off the gas supply as soon as the flame is extinguished. Koch's lamp, belonging to this type, works in the following manner: a metal tongue consisting of iron and brass sheet and bent downwards projects into the flame. The lower end is bent round and acts as fulcrum for a lever loaded with a weight. If the flame is extinguished by any cause, the metal tongue cools and approaches the burner; by this motion the lever loses its support and takes up a vertical position, and by so doing turns off the gas.

Thermometers.—It is convenient to use thermometers about 10 cm. long with a maximum reading of 25° C. for measuring temperatures in thermostats. They can be fixed in a bored cork which has small grooves down the sides and rests in a wide-mouthed glass vessel. The air in this communicates with that in the thermostat through the grooves on the cork. If the thermometer is fitted up in such a glass, it can be taken out and the temperature read without fear of its changing, as would be the case if the thermometer alone were removed from the warm thermostat. It is not out of place to remember that ordinary thermometers cannot always be depended on, but must be checked before they are used. It is also necessary to do this from time to time, as the thermometers change in course of time. A thermometer is checked by comparison with a standard thermometer, the zero point of which has been exactly determined previously by immersing its bulb in finely pounded ice. A possible error is thus removed.

5.—*Sterilising Apparatus.*

With Dry Heat.—Glass and metal apparatus are sterilised by means of dry heat. This is done by using an oven made of iron, coated with lead and of the shape shown in Fig. 22. It consists of a double-walled chamber of cylindrical form ; the outer wall is coated with asbestos on the inside and is conical at its lower end; a large gas burner projects into the conical part, so that the products of combustion rise between the walls and leave the apparatus by the chimney on the top. The door is also double walled. The burner can be raised or lowered, its position and the proper size of flame being determined by experiment.

There is quite a large number of different designs of these hot air chambers. The principal point is that they must give a temperature of 150° C., and must be durable. A regulator may be used along with them, but it is not necessary in most cases since it is immaterial whether the glass articles are heated to a little more or a little less than 150° C. On the other hand, if the hot air chamber is used for sterilising the gypsum blocks to be referred to later, it is of great importance that these should not be submitted to a higher temperature than 120° C., because they lose water of crystallisation and afterwards crumble on being placed in water. The simplest way, however, is to use an iron box, the walls

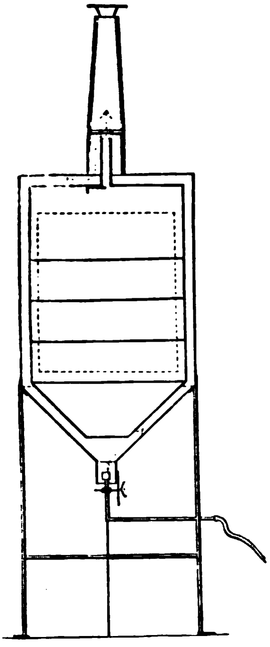

FIG. 22.—Apparatus for Sterilisation with Dry Heat.

of which are coated with asbestos paper and which is provided with a regulator. An ordinary Bunsen burner is sufficient to obtain temperatures not exceeding 120° C.

Culture media are sterilised by boiling on a sand bath or by means of steam. A sand bath is best made out of a shallow rectangular iron box on four feet. Below the box are placed two horizontal and parallel iron tubes closed at one end, connected with the gas supply and pierced on the top with holes which serve as burners. At the end of the tube where the gas enters there are holes like those on a Bunsen burner. The best dimensions for such a sand bath are, length about 42 cm., breadth about 22 cm. On the above there is room for 10 to 12 ⅛-litre Pasteur flasks.

It is simpler however to sterilise flasks containing culture media in an autoclave to be described later. Sterilisation by steam is often necessary and is the most frequent means employed.

Steam Sterilisation.—An autoclave or digester is quite indispensable for this purpose, and with it sterilisation can be effected with or without pressure; an ordinary steamer cannot be used with pressure. The accompanying sketch (Fig. 23) shows a Chamberland autoclave made by Wisnegg of Paris. It consists of a steel vessel with a cover which can be closed tightly and screwed down. On the cover there is a tap, *b*, by means of which the steam may escape if no pressure is required, a safety valve, *a*, and a manometer, *c*. Below the vessel there are two concentric rings of gas burners, and the whole is surrounded by a jacket of sheet iron provided with a door. In an autoclave, the height of which is 60 cm., the inner space used for containing the objects to be sterilised is about 32 cm. deep, and is divided up by several horizontal partitions.

When the autoclave is about to be used, distilled water is put into the vessel, the amount depending on the size of the latter. The objects to be sterilised are thereupon placed on the shelves and the cover screwed down. All the gas flames are then lit, and after the water in the vessel begins to boil, the outer ring of flames is extinguished. If no pressure is required, the tap on the cover remains open during the boiling, otherwise it remains closed. The pressure is indicated by the manometer and is regulated by the

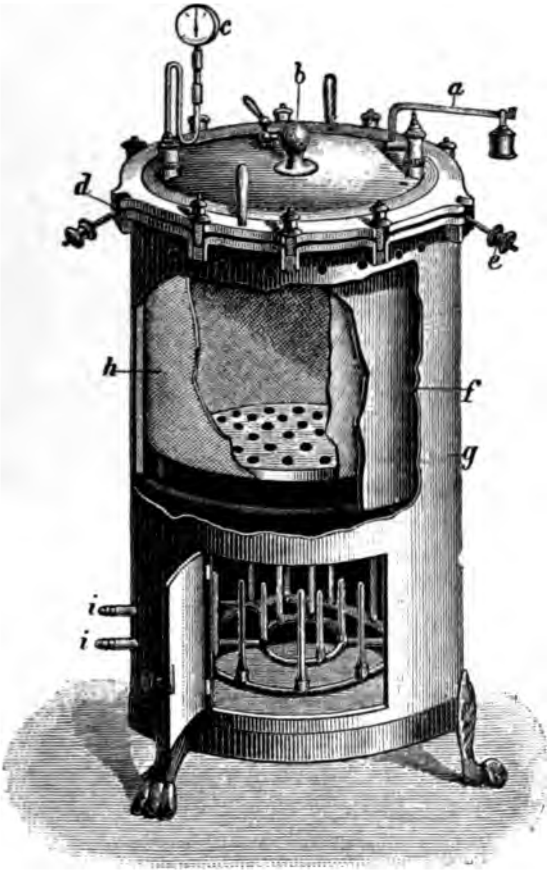

Fig. 23.—Chamberland's Autoclave.

source of heat. If the liquids to be sterilised froth strongly during the boiling, only one ring of burners is lit; the heating then proceeds more slowly and frothing is avoided.

As mentioned above, an ordinary steamer can be used for sterilisation without pressure, that is, for simply heating by means of steam. It is fitted up in practically the same manner as an autoclave, only it need not be so strong, and the valve and manometer are omitted.

A steamer of this kind used in the Carlsberg laboratory is made of tinned copper, is 1 metre high and has an inner diameter of 42 cm. The inner space for holding the objects to be sterilised is 65 cm. deep and is divided into three stages by perforated platforms. It is charged with 5 litres of distilled water.

6.—*Culture Vessels.*

We shall now describe various flasks and culture vessels. After all the openings have been closed with cotton wool such vessels are sterilised in a dry heat for two hours at 150° C., this being the usual mode of treatment of glass ware. They must be tolerably dry before being put into the steriliser.

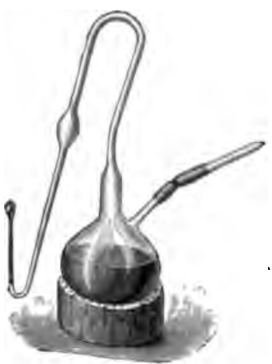

FIG. 24.—The Hansen Modification of the Pasteur Flask.

The Pasteur Flask.—We are indebted to Hansen for the improved modification of this flask now in use (see Fig. 24). The flask consists of a glass bulb drawn out into a long tube wide at the beginning, bent twice and with a bulb between the two bends. An inoculation tube, a short straight side tube, proceeds from the bulb, and is closed with a rubber tube and glass rod. The Pasteur flask is commonly used in three sizes, having capacities of $\frac{1}{5}$, $\frac{1}{2}$ and 1 litre respectively. As the bottom of the flask is not flat as a rule, a ring of cork or pasteboard (see Fig. 24) is used as a support.

It is specially important that all the Pasteur flasks, small and large, in the laboratory should have side tubes of the same diameter as those on other vessels to be referred to later; otherwise the tubes do not fit into the same rubber connections when the flasks have to be joined with one another. Standard flasks ought therefore to be kept as patterns for new stock, and in giving an order, the external diameter of the side tube should be given exactly; 6 mm. is a suitable diameter.

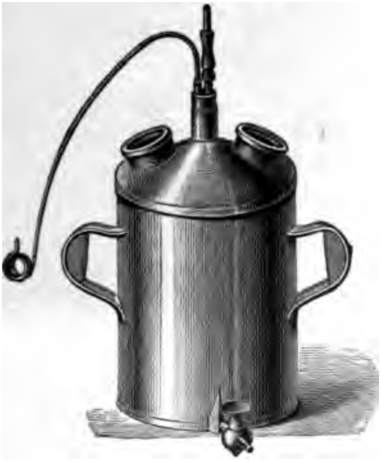

FIG. 25.—The Pasteur Vessel.

When Pasteur flasks containing culture solution either alone or with cultures are put aside, the bent tube should be closed with a small asbestos plug which filters any air that may penetrate into the flask as the result of temperature changes. When flasks are placed in a very cold situation, and therefore usually a very damp one, salicylic cotton wool should be used instead of asbestos, as moulds cannot grow through the former. These flasks have the great advantage of only allowing a very slight evaporation of the liquid; they may remain for many years without any notable evaporation occurring. Thus Pasteur flasks with cultures have been standing in the Carlsberg laboratory for twenty years during which no appreciable diminution of their contents has taken place.

Hansen introduced the above-mentioned expansion of the bent tube, in order to avoid infecting the culture medium by germ-laden air bubbles being carried into the flask itself as a consequence of the sucking back which takes place after boiling. These bubbles are caught in the little bulb, and deposit their germs on the glass. The tube and bulb should, therefore, be always heated to redness before beginning to work with the flask, in order to kill the germs which may have settled there.

The Carlsberg Vessel.—Large culture vessels are made of metal, *e.g.*, tinned copper, as it is not convenient, on practical grounds, to use glass flasks of more than 1 litre capacity, and because, in general, vessels containing 7 to 8 litres of nutrient solution are used in preparing pure yeast for brewing purposes. Pasteur was the first to use a vessel of this kind, as shown in Fig. 25, for carrying on fermentation experiments. It was closed by a two-holed rubber bung fitted with a short straight tube closed by a rubber tube with glass stopper for introducing the yeast, and a long bent tube for allowing the carbonic acid generated during fermentation to escape. On the top there were two windows, and near the bottom an ordinary metal stopcock for drawing off the liquid and yeast.

But this form had somewhat great disadvantages, as it was found that the contents of the vessel were often infected through the windows and stopcock, it being very difficult to keep the latter sterile. On this account Hansen and his assistants gradually evolved the Carlsberg vessel described below. There are still some who prefer the Pasteur vessel for its window and tap; it is, therefore, not out of place to indicate its failings.

There are two modifications of the Carlsberg vessel, an older, cheaper form, and a newer, more expensive one; they are shown in Figs. 26 and 27. There are two straight side tubes on this vessel, one, *a*, on the top, and the other, *b*, a little above

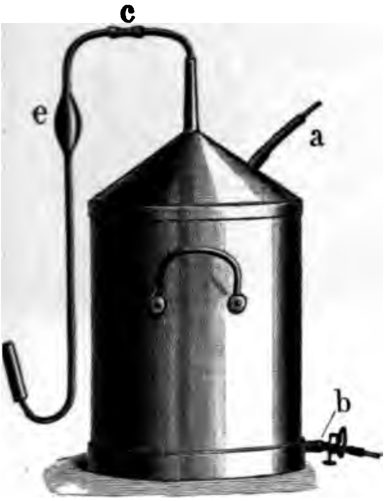

Fig. 26.—The Carlsberg Vessel, old model.

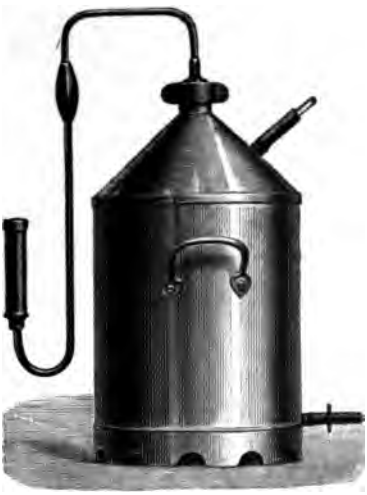

Fig. 27.—The Carlsberg Vessel, new mod

the bottom; the bent tube is fitted either to screw off (see Figs. 27 and 28), so that both vessel and tube can be easily cleaned, or a part of it is fixed to the vessel and the other part connected by means of a piece of rubber tubing, *c* (see Fig. 26). The first-named form is the more expensive, as has been remarked, but it is to be preferred. The bent tube, at the end of which a filter, *d*, can be fitted, is expanded in the middle, *e*. This filter was in the older model a glass tube filled with cotton wool; in the new model it consists of a metal cylinder filled with asbestos, provided with a loose-fitting top, and screwed on to the tube. Rubber tubes are fitted to both of the side tubes, and are closed by glass stoppers; the rubber on the lower side tube is also provided with a pinchcock. The yeast is introduced into the liquid in the vessel through the upper side tube, the liquid and yeast being removed through the lower one. The chief purpose of this vessel is the growing of yeast in large quantities, as, for example, when such is required for the pure culture apparatus mentioned later; it is also used for fermentation experiments with larger quantities of liquid.

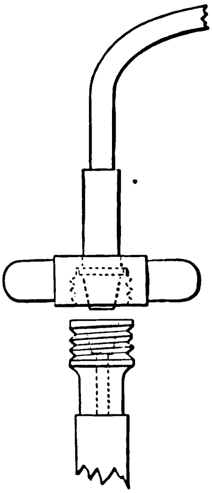

FIG. 28.—Mode of connecting the Bent Tube with the Carlsberg Vessel.

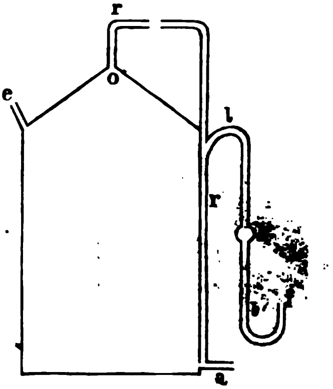

FIG. 29.—Prior's Vessel.

The manner of connecting the bent tube with the vessel in the new model may be seen from Fig. 28. The tube fits into the vessel by means of a conical joint; the connection is effected by means of a female screw. This joint must be made very accurately, otherwise the flask is useless.

The most convenient capacity for this vessel is about 10 litres. The largest amount of wort with which such a vessel can be charged is from 7 to 8 litres if it is to be used for fermentation experiments, for it must not be completely filled. In general 125 to 150 grams of thick yeasty sediment can be grown in the above quantity of wort.

Sometimes it happens that the bent tube becomes blocked during the sterilisation of the wort, so that it is impossible to draw off the contents of the vessel. This difficulty is easily surmounted by passing into the tube a thin, doubled copper wire. As a rule little is gained by heating the tube, since it is not known at what point the tube is stopped. It is advisable, now and then, to boil out the Carlsberg vessels with solution of soda, to remove the hop resin, etc., clinging to the sides.

Prior's Vessel.—E. Prior has constructed a modification of the Carlsberg vessel, which is represented in Fig. 29. A side tube, *e*, is closed with rubber and glass stopper; the two tubes, *r*, are connected by means of a piece of rubber tube carrying a pinchcock, and the tube, *a*, is also provided with a short rubber tube and pinchcock. The filter is fitted on at *f*. This modification of the original model has been designed chiefly to allow aëration of the wort. For further information see page 77.

The Chamberland Flask and its Modifications.—As is to be seen from Fig. 30, this flask consists of a flat-bottomed bulb with a short neck provided with a ground cap which is drawn out into a somewhat long tube filled with cotton wool.[1]

A more frequently used modification of this flask is that known as the Freudenreich flask. It (see Fig. 31) differs from the Chamberland flask in being cylindrical. As regards the size of the flask a total height of 10 cm. and a length of tube 2 cm. may be recommended. The external diameter of the base is about 2·5 cm., the internal diameter of the neck about 1 cm. The inner diameter of the tube ought not to be more than 0·2 cm., and the tube itself should not be shorter than the length mentioned above, otherwise too great an evaporation of the nutrient medium takes place. A flask like this holds about 20 c.c.; but, as a rule, it should not contain more than 10 to 15 c.c. of liquid.

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[1] Ordinary cotton wool (not fat free) should always be used for plugging flasks. Fat-free cotton wool attracts moisture and can thus set up infection.

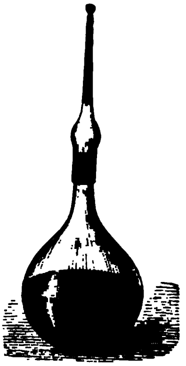

FIG. 30. —The Chamberland Flask.

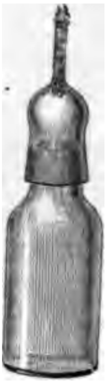

FIG. 31.—The Freudenreich Flask.

This flask is very frequently used, as it does not take up much room, and on account of its small size also, working with it is not expensive. But the flask has this failing that it is somewhat easily upset. It is therefore advisable to place these flasks in small tin boxes made of various sizes, *e.g.*, for 6, 10, 15, 25, 50 and 100 flasks. The height of the box may be made 3·5 cm.; the other dimensions are, corresponding to the above numbers: 6 × 9 cm., 6 × 14 cm., 9 × 14 cm., 14 × 14 cm., 14 × 28 cm. and 19 × 38 cm.

When using the Freudenreich flask, care must be taken the cap fast ; the flask ought never to be lifted by , for it may easily happen that the cap comes off in d, and the growth in the flask is thus infected.

e Hansen flask forms another modification of the berland model (see Fig. 32). It is distinguished by g a side tube, the lower part of the flask being either lar or cylindrical. The flask has this advantage, that n be connected with the side tube of the Pasteur flask.

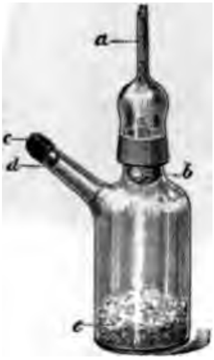

FIG. 32.—The Cylindrical Hansen Flask.

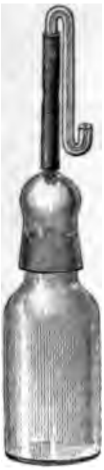

FIG. 33.—A Freudenreich Flask with Rubber Tube and an S Tube.

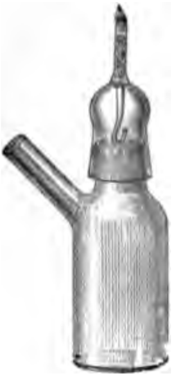

FIG. 34.—The Jörgensen Flask.

The side tube is either closed with an asbestos plug and sealing wax, or with a rubber tube and glass stopper. The first method is used specially when cultures are to be kept a long time in such flasks.

Freudenreich and Hansen flasks are used not only for cultures in or upon culture liquids, but also for cultures on nutrient gelatine, which is allowed to solidify obliquely in order to obtain a larger surface. Although the evaporation is but small in Freudenreich and Hansen flasks, it is advisable, when the latter have to remain a long time with cultures on gelatine, or in or upon liquids, to attach a small S-shaped glass tube to the cap by means of a short rubber tube (Fig. 33). During an experiment the tube and rubber are removed from the flask. If the flasks only contain nutrient gelatine or liquid without cultures, the mouth of the cap tube may be closed with a little sealing wax to prevent drying up.

For this purpose Alfr. Jörgensen has fitted a small hook-shaped tube to the cap of the Hansen flask (see Fig. 34).

The Freudenreich and Hansen flasks are well adapted for sending yeast specimens, the latter especially, as the culture liquid can be directly transferred to it from another flask provided with a side tube. Fig. 32 represents such a flask on the bottom of which a layer of fat-free cotton wool, *e*, with yeast is placed, and the side tube of which is closed with asbestos and wax, *d* and *c*. The neck of the flask has also a cotton-wool plug, *b*, the tube of the cap being filled in the ordinary way with cotton wool, *a*.

In order to prevent interchanging of caps in cleaning, both flask and cap should be etched with the same number.

Pipettes must be used in working with culture liquids in Chamberland and Freudenreich flasks.

Flasks.—For many experiments, the ordinary Erlenmeyer flasks (with a capacity of 200 to 250 c.c.) are very suitable because they can be used for solid as well as liquid nutrient media. The layer of medium has a large surface, so that air has free access to the cultures. Fig. 35 represents one of these flasks which is seen to have a tolerably wide neck which is closed by a cotton-wool plug tied down with a double layer of sterile filter paper.[1]

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[1] When flasks covered in this way are to be put away in a damp place, a mould growth soon forms on the filter paper and the string which ties it down. This can be avoided by using a filter paper impregnated with a

The cotton-wool plug must always be passed through a flame before and after inoculation to kill any germs on it.

As soon as the plug is quite dry after sterilisation, but not before, it is advisable to replace the double layer of filter paper by a tightly fitting rubber cap after the upper portion of the cotton-wool plug has been sterilised in the flame, in order that the culture medium may not completely dry up when left for a long time.

Globular flasks, not having such wide necks, are better adapted than the Erlenmeyer flasks for preserving small quantities of culture gelatine which are to be used for plate cultures.

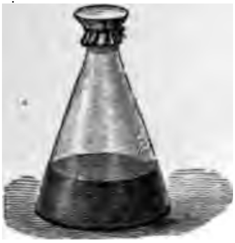

FIG. 35.—The Erlenmeyer Flask.

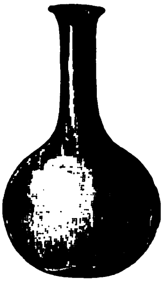

FIG. 36.—Globular Flask.

The size in general use has a capacity of 80 to 90 c.c. and is charged with about 15 c.c. of gelatine. It is not advisable to use smaller flasks than these, as the melted gelatine cannot be shaken up vigorously enough.

The form of the globular flask may be seen in Fig. 36. It is closed with a cotton-wool plug and a double layer of filter paper. If the gelatine is to stand for a long time, a rubber cap may then be used instead of the filter paper, the same precautions being observed as were described above.

Of the flasks already described the Pasteur flask is the

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10 per cent. alcoholic solution of salicylic acid and then dried. The string can be similarly treated.

most reliable one to work with. This flask is expensive and takes up much room; though not standing so well, the small cylindrical flasks, being cheaper, are coming into use wherever suitable. Test tubes are also used in many laboratories; but they are less reliable and are not well adapted for cultures in nutrient liquids.

In wine-manufacturing establishments large glass flasks with double-bored stoppers are used instead of the Carlsberg flask. Two tubes are fitted to the stopper as in the Pasteur flask.

Petri Dishes.—Petri dishes consist of a set of two flat glass dishes (see Fig. 37). A suitable diameter for the lower dish is about 9 cm. and for the upper 10 cm., the height being about 1·5 cm. These dishes are used for plate cultures. Each set is wrapped in a double layer of filter paper and sterilised.

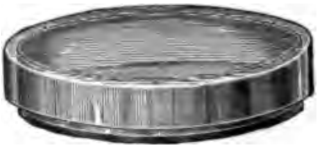

FIG. 37.—Petri Dishes.

7.—*Apparatus for Spore Cultivation.*

Gypsum Blocks and their Containing Vessels.—Blocks of gypsum are generally used for the cultivation of the spores of saccharomycetes. Fig. 38 represents a block of gypsum placed in a covered glass dish with sterile water. The block is in the form of a truncated cone, and the cover of the vessel fits quite loosely. The dishes used in the Carlsberg laboratory are the so-called "bird troughs" (Vogelnäpfe). A suitable size for these, taking outside measurements, is as follows: Height, 4·5 to 5 cm.; diameter of the bottom, about 7 cm. The gypsum block is 3 cm.

high; the diameter of the lower surface is 5·3 cm., that of the upper surface 3·8 cm. The manner in which the culture is made on the gypsum block will be described further on. When "bird troughs" are used which have a somewhat convex bottom, the gypsum block must be concave underneath. To make a gypsum block, 2 parts of powdered gypsum are mixed with ¾ part of water and the mixture poured into a tin mould. The block should be hard, and the mould must not be rubbed with fat, oil or such material. Sometimes glass plates are used instead of glass lids, but the latter are to be preferred.

A culture on a gypsum block in such a vessel cannot, as a rule, be kept free from bacterial infection, for the cover must not be tightly closed down, but should allow free access of air. When therefore a spore culture is to be preserved in a pure state, one uses not gypsum, but a shallow layer of water in a culture flask; these and other methods are described later. But as a richer spore formation is got in the cultures on gypsum blocks than on other substrata, it is sometimes of importance to be able to produce bacterium-free spore cultures on gypsum blocks. Schiönning has therefore described a method for doing this. As may be seen in Fig. 39, there is taken for this purpose a Hansen flask into which the gypsum block has been moulded. The procedure is as follows: First a paper cylinder is made with a diameter a little smaller than that of the neck of the flask.

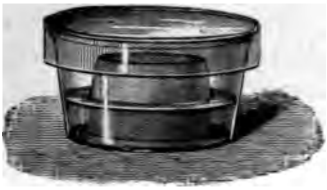

FIG. 38.—Gypsum Block in a glass dish with water.

This paper cylinder is used as a mould, and a mixture of 2 parts of gypsum and ¾ part of water is poured into it. A glass rod is used for stirring the mixture to drive out air bubbles. After the gypsum has solidified the paper is taken off, a suitable length of the gypsum cylinder cut off, and a shallow depression made at both ends. The upper depression serves later for holding the yeast, the lower one enables the cylinder to stand better on the curved bottom of the flask. As soon as the cylinder has been placed inside the flask it is fixed in position by some of the gypsum paste being cautiously poured on the bottom of the flask through a small paper filler without disturbing the position of the cylinder. The side and top tubes are closed with cotton wool.

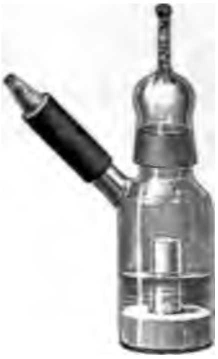
FIG. 39.—Gypsum Block in a Hansen Flask (after Schiönning).

These flasks and the ordinary glass dishes with gypsum blocks are sterilised for 1 to 1½ hour at 110 to 115° C., the glass dishes being first wrapped in a double layer of filter paper. As mentioned above, the temperature should not rise above 120° C. The ordinary gypsum blocks and cylinders are sterilised in a moist condition, but are difficult to get perfectly sterile. Spores of bacteria clinging to them will often survive the heating.

The rubber tube fitted on the side tube in Fig. 39, which is closed with a glass tube filled with cotton wool, is only fixed on after the culture is laid on the block. The tube is at the same time used for connecting with another flask holding sterile water.

The large gypsum block standing in a vessel, as described above (Fig. 38), is used for spore cultures in nearly all cases, and is extremely convenient. The blocks of gypsum can be used several times. They are cleaned by immersion in water; if very much contaminated they are boiled, being afterwards brushed with a stiff brush, and finally shaved with a piece of glass or something of the kind, after which they are washed with water.

Sterile Water Holder.—A holder with sterilised water is brought into use in many kinds of experiments, for example, in gypsum block cultures. Fig. 40 represents a holder made in such a way that some of the water can be drawn off without the remainder being infected.

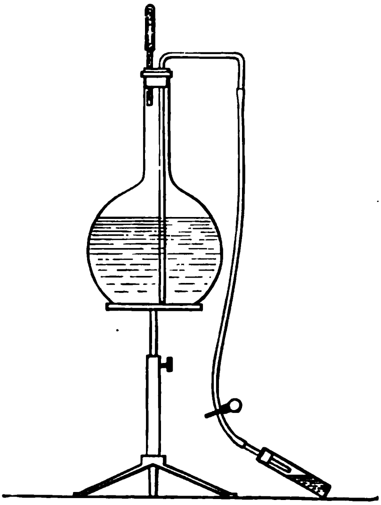

FIG. 40.—Holder for supplying Sterile Water.

The apparatus consists of a globular or conical flask with a capacity of about 2 litres. It has a double-bored rubber stopper. Through the one hole a glass tube passes to the bottom of the flask; it is twice bent outside and a rubber tube with a pinchcock is attached to the end. A filter, which consists of a glass tube packed with cotton wool and covered with a loose glass cap, is placed in the other hole of the stopper.

The whole apparatus is sterilised in the following way: The flask is filled with the necessary amount of distilled water, the stopper put in place and the long glass tube adjusted so that its end is above the water surface. The hole in which the filter is to be placed is shut by means of a glass stopper and the pinchcock removed from the tube. The water is now boiled on a sand bath for an hour, the steam passing freely through the glass and rubber tubing. The flask is now removed from the sand bath, the glass tube pushed down to the bottom, the glass stopper removed and replaced by the sterilised filter. The apparatus is again placed on the sand bath and the water again made to boil; on account of the increasing pressure the water is now driven through the glass and rubber tubing; the pinchcock is now fitted to the latter and the flask removed from the sand bath. All that is required now to get a continuous stream of water is to open the pinchcock, the tube acting as a syphon. To prevent the apparatus from becoming infected when not in use, the glass jet at the end of the rubber tubing is passed through the cork of a test tube or small flask containing alcohol. Before use, a little water is allowed to run off to remove alcohol. The whole apparatus is placed on a stand which should not be too low. In more delicate experiments it is preferable to use sterile water taken from such flasks as are only opened once.

8.—*Moist Chambers.*

Moist chambers are employed in investigations on development, for producing pure cultures, etc.

Hollow Glass Slips.—The simplest form of this apparatus is the hollow glass slip, by which is understood a slip having an oval or circular depression in the middle. A drop of culture medium or liquefied nutrient gelatine containing the micro-organism to be examined is placed on a cover glass and laid over the depression so that the drop is below. The cover glass is then fastened to the glass slip with vaseline.

Ranvier's Moist Chamber. — We may use Ranvier's moist chamber for the same purpose (see Fig. 41). It consists of a glass strip in which there is an annular groove. The medium containing the organism is placed on the part of the glass slip inside the groove, which is thinner than the outer part of the strip; a drop of water may be put into the groove, this however not being always necessary. A cover glass is now carefully laid on so that the drop does not flow into the groove. To keep the cover glass in position, it is smeared with vaseline, which may be applied in the liquid condition by means of a brush. The cover glass is thus made to fit air-tight. In addition the edge can be painted with a liquid mixture of 1 part of vaseline and 2 parts of beeswax.

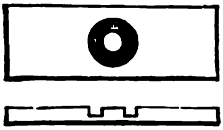

FIG. 41.—Ranvier's Moist Chamber.

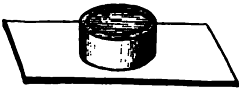

FIG. 42.—Böttcher's Moist Chamber.

Böttcher's Moist Chamber. — Fig. 42 represents Böttcher's moist chamber in which a glass ring is cemented to a glass slip.[1] A drop of water is placed on the bottom of the chamber, and the cover glass carrying the culture medium and the micro-organism next laid on the ring. The culture is, of course, on the under side of the cover glass, the latter being fixed on the glass ring by means of melted vaseline. The cover glass may also be stuck on to the ring by means of fish glue and then the ring fastened to the glass slip by means of vaseline. The ring must be adjusted on the glass slip so that the adherent medium is not dissolved by the water. In most cases only one drop of water is placed in the middle of the chamber, so that even when the adherent substance is soluble in water, there will be little chance for the ring to become loose. It is certainly best to use an insoluble medium when a loose adhesion with vaseline is not employed. An adhesive medium can be made from a mixture of wax and a little turpentine; it is painted on after melting. Syndetikon, fish glue, water glass, etc., are all soluble in water.

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[1] Good strong chambers of this kind may be obtained from Messrs. Altmann, Berlin.

Two sizes of rings are in general use, having diameters of 30 mm. and 18 mm. respectively. The former are used chiefly for preparing pure cultures, where the presence of a large number of colonies is desired in one chamber. Squared cover glasses are often used for this.

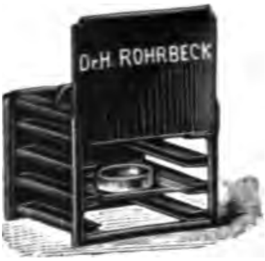

FIG. 43.—Stand for Moist Chambers.

Stand for Moist Chambers.—When many moist chambers with cultures are in use at the same time it is convenient to have a stand for them (see Fig. 43).

9.—*Additional Apparatus.*

Pipettes are used in many sizes and it is necessary to have a large stock of them. Some of these must be graduated and have a capacity of ¼ c.c. to 100 c.c. It is also necessary to have ready various sizes of ungraduated pipettes, a number of them being drawn out at one end into long capillary tubes. Some of each kind must have the lower part long and thin enough to reach to the bottom of the Pasteur flasks through the side tubes. Such pipettes as a rule are not on sale, so that they have to be ordered from a glass blower. Before the pipettes are sterilised, the top end is closed with a small plug of cotton wool. They are sterilised and kept in suitable metal cases provided with lids and on the bottom of which some cotton wool is placed; or each one may be wrapped in filter paper and sterilised.

Rubber Tubing for Flasks.—Red rubber tubing is used for culture flasks having side tubes. Pieces 8 to 9 cm. long will be found the most suitable for the Pasteur flasks. These are first washed in spirit and then both ends are closed with glass stoppers, the latter having a length of about 6 cm. and being drawn out at both ends. To prepare a number of such tubes in sterile condition they are placed in a beaker or similar vessel, covered with filter paper tied on, and kept in a current of steam for one hour. After the rubber tubes have been once used they must be boiled in water before sterilisation.

Platinum Brush.—Lastly, platinum brushes ought to be mentioned here, these having often proved useful. They consist of pieces of fine platinum wire which are collected together like the hairs of a brush and fused into the end of a glass rod. They may be obtained from Geissler's successors, Bonn.

II.—NUTRIENT MEDIA.

We shall now describe the nutrient media commonly employed, their preparation and sterilisation.

1.—*Liquid Media.*

Beer Wort is one of the most frequently used nutrient solutions. It is not usually prepared in laboratories but obtained from breweries, and may be used either as malt wort or as ordinary hopped wort; the former, however, is only exceptionally used. If a clear wort is desired, filtered wort, *i.e.*, wort which has been passed through the filter bags in the brewery, can be used. An absolutely clear wort is obtained by setting this aside and allowing it to take up oxygen from the air; a deposit forms at the same time.

If the method of boiling on the sand bath is employed for sterilisation of the wort, the latter must be diluted with sterile water on account of the great evaporation which takes place, so that the concentration may be approximately the same after sterilising as before; usually a mixture of 7 parts of wort and 1 of water will be found most suitable. The dilution is unnecessary when steam heating is used for sterilising.

Wort contained in Pasteur flasks is sterilised on the sand bath in the following manner: The wort is boiled for an hour; during the first three-quarters of an hour the steam is allowed to pass out of the side tube through the open rubber tubing; the glass plug after being sterilised in the flame is then put into the tube so that during the remaining time the steam only passes through the bent tube. As soon as the flask is removed from the sand bath, the tube is closed with the asbestos plug. The air which passes in as the flask cools is sterilised by its passage through the hot tube. Later on, after the tube has cooled, the current of air passes very slowly through the tube and is thus filtered by the asbestos.

The wort in the different flasks is sterilised by means of steam at ordinary pressure for a half to three-quarters of an hour, large flasks with 70 c.c. of wort requiring three-quarters of an hour, the smaller flasks, *e.g.*, the Freudenreich, half an hour.

After sterilisation it is advisable to let the flasks remain at least fourteen days before use in order to be able to judge to what extent the wort is sterile. During this time the wort takes up oxygen, and the germs which may possibly be present will develop.

Sterilisation of wort in Carlsberg vessels is performed in the following way: Each of the two straight side tubes is provided with a 10 cm. length of thick grey rubber tubing which must be very stout and fit exactly. To prevent displacement it is fastened with copper wire. The asbestos filter, the lower opening of which is closed with cotton wool (the upper one has a loosely fitting metal cover), is sterilised by itself in filter paper in the hot air chamber for two hours at 150° C. A glass plug is inserted in the lower rubber tube which is also provided with a pinchcock. After this, about 5 litres of distilled water are poured into the vessel, which is then placed over a powerful gas flame. The water is kept boiling for three-quarters of an hour, during which time the upper side tube remains open. The hot steam thus passes out, until at the end of three-quarters of an hour the tube is closed with a glass plug; steam is then allowed to pass for a quarter of an hour through the bent tube, the gas flame being meanwhile turned down a little; afterwards the hot water is allowed to run out through the lower side tube and sterilise it, in case the vessel should be used the same day for sterilisation of beer wort. If this is not the case, only about 100 c.c. are drawn off, the rest remaining in until the vessel is filled with wort, the bent tube being closed with an asbestos plug. While the water is running out, the bent tube is heated with a gas flame to sterilise the air which passes in to replace the water. When the water has been removed in this manner, the vessel can be filled with nutrient solution. We will assume that this, as is usually the case, is wort. The vessel is then charged with 7 litres of ordinary wort and 1 litre of distilled water,[1] the asbestos plug being taken out of the bent tube and the mixture heated to boiling; the glass stopper is also removed from the upper side tube. The wort is boiled for three-quarters of an hour. The rubber of the upper side tube is then closed with a glass plug which has been sterilised in the flame, and the steam allowed to escape for another quarter of an hour through the bent tube, under which a gas flame is now placed. About 100 c.c. of boiling hot wort are then drawn off through the lower side tube, and the rubber tube thus sterilised again. The part of the rubber outside the pinch-cock is cleaned either with the aid of sterile filter paper or by means of a hot iron rod, whereupon the glass plug previously sterilised in the flame is quickly put into place; during this the flame is kept under the bent tube. As soon as the wort has become lukewarm the filter is placed on the bent tube. To do this it is taken out of the filter paper in which it was wrapped for sterilisation, the cotton-wool plug is removed in a flame and the filter screwed on to the tube, the Bunsen flame meanwhile being held over the mouth of the latter. Finally the flame used for heating the bent tube is removed.

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[1] This amount is sufficient when a three-burner Bunsen is used for heating the vessel.

Before the wort is used it must be aërated. This can be done by allowing the wort to stand for several months. If, however, an air-pump can be used the aërating and cooling of the wort can be performed on the same day. Assuming that the air from the pump is pumped through a tube which ends in a stopcock, the procedure is as follows: Before the air comes into contact with the wort it must be free from stray germs and is therefore passed through a filter; the latter consists of a metal tube filled with cotton wool, and is conveniently about 25 cm. long and 3 cm. in diameter. About 40 grams of cotton wool are necessary for filling the tube, and should be sterilised at 150° C. for two hours previously. The whole filter is then packed in filter paper, sterilised in the same way and then connected with the tube from the air pump.

While the wort in the vessel is still boiling, a gas flame is placed under the bent tube through which the steam is escaping. The rubber tubing of the lower side tube is then connected with a bent sterilised glass tube, and the latter placed in connection with the air filter by means of a sterile rubber tube.

The air stopcock above the filter is now opened a little, and at the same time the pinchcock, which is afterwards pushed forward over the rubber tubing on the glass tube; the air now goes through the wort. The gas flame under the vessel is then removed, and some minutes later the flame also under the bent tube. The aërating is continued until the wort cools to 30 to 35° C., *i.e.*, for about five to six hours. If the temperature during aëration falls below 30° C. the wort usually froths out through the bent tube, and this, of course, ought to be avoided. About 60 litres of air are required for 7 to 8 litres of wort.

Before the aëration is stopped the bent tube is again heated. The communication is now interrupted by closing the lower rubber tube with the pinchcock, and at the same time the stopcock above the air filter. The glass tube is removed from the rubber tube, the latter cleaned out and speedily closed with the flamed glass plug. Simultaneously with the removal of the gas burner from under the bent tube the asbestos filter is screwed on. As soon as the wort is completely cooled it is ready for use; yet it is also advisable here to let it stand for fourteen days in order to make sure that it is really sterile.

Besides its use in fermentation experiments with large quantities of liquid, the Carlsberg vessel can also be employed with advantage in storing large quantities of nutrient solution; the latter can then be drawn off into smaller flasks which are connected with the large vessel. This is of importance, for example, when one requires an absolutely clear wort: it is then only drawn off from the Carlsberg vessel after it has stood long enough. If the same wort is to be placed in a larger number of flasks the same procedure is followed. While tapping, the filter is removed and the bent tube heated.

When it is expedient to avoid sterilising brewery wort again in the laboratory, the Carlsberg vessel may also be used by putting it in communication with the wort cylinder of the Hansen-Kuhle pure culture apparatus, and filling it with the cooled, aerated, sterile brewery wort. For many experiments this has, in addition, the important advantage that exactly the same wort can be treated in the laboratory as is treated in the brewery; the composition of the wort is, of course, very much changed by repeated sterilisation.

When, therefore, the wort from the wort cylinder is to be filled into a Carlsberg vessel the following process is adopted: The upper side tube of the vessel is connected with a cock on the wort cylinder by sterilised glass and rubber tubing. This manipulation must, of course, be performed with due regard to all precautions, all the more as the wort cylinder is generally set up in the neighbourhood of the fermenting room, and the conditions of working are thus more difficult than in the laboratory. A right-angled glass tube is first inserted in the rubber of the lower side tube of the vessel (the pinchcock remaining in position); this tube should be about the height of the vessel, and should have been sterilised beforehand. The advantage in using this glass tube is that the quantity of wort in the vessel may be noted and the supply cut off when the vessel is exactly full. When the vessel has been connected with the wort cylinder in this fashion the asbestos plug is removed from the bent tube and the wort is allowed to flow, the pinchcock on the lower side tube being at the same time opened. When the vessel has been charged with the required quantity of wort the pinchcock is shut, and immediately afterwards the tap of the wort cylinder. Before communication between the vessel and the cylinder is interrupted the sterile filter is screwed on to the bent tube of the vessel in the usual manner. Then the glass tubes are taken out of the rubber of the two side tubes, this being done in the flame. Before the flamed glass plug is inserted in the lower rubber tube the latter ought to be sterilised by means of a hot iron rod. When the right-angled glass tube is removed the wort remaining in it runs out, and the rubber tube of the Carlsberg vessel may thus be wetted. If this happens it must be well washed with spirit and afterwards flamed, dried up wort being a splendid culture medium for moulds and other micro-organisms.

If a Prior vessel is used, the aërating of the wort proceeds as a consequence of the temperature difference between the air in the vessel and that outside, the air being sucked through the wort. The construction of the vessel may be seen from Fig. 29.

As soon as the wort in the vessel is sterilised in the usual way and some hot wort drawn off through *a*, thus sterilising this tube, the flame is extinguished, the air filter, *f*, put in place and the rubber tubing connecting both parts of the tube, *r*, completely closed by a pinchcock. The air which is sucked in at *a*, in consequence of the formation of a rarefied space over the wort, passes through the filter at *f* into the lower part of the tube *r*, then through *a* into the wort, and collects in the flask above the wort, which takes up more or less of its oxygen. The aërating is continued until the wort has reached the temperature of the surrounding air, and is very vigorous in the first stages, as one can hear from the noise of the bubbling. As soon as the temperature has reached a state of equilibrium the pinchcock is opened, thus making communication with the outer air again in the old way. Cultivation of yeast can be proceeded with immediately.

Wort, with the addition of tartaric acid (to the amount of 0·3 per cent. for mixtures of yeast and bacteria to exclude the latter), is made by adding a concentrated solution of tartaric acid in sterile water to the necessary amount of wort ; the mixture is then sterilised. There now arises this difficulty, that a fairly strong deposit forms, containing, among other things, albuminoids ; both solutions can, therefore, be mixed after sterilisation, when only a small proportion of tartaric acid is added ; the dilution of the wort is then so slight that it may be neglected. When it is desired to make a specially strong solution of tartaric acid in wort the acid is dissolved directly in the wort. Such tartaric acid solutions bump violently during boiling ; this may be avoided by adding to the solution some pumice stone which has been heated to redness.

Water is invariably used distilled, and is tolerably difficult to obtain completely sterile ; it ought, therefore, to be always sterilised several times at intervals of one to two days in a current of steam. Sterilising two or three times is sufficient in most cases : the water is then boiled for three-quarters of an hour the first time, and for half an hour the second and third times. The reason for proceeding in this manner lies in the fact that certain bacteria spores present in water can survive a temperature of 100° C., while, on the other hand, the growing cells of these bacteria are destroyed at this temperature. The germinating power of the spores can even be increased by heating ; the water is, therefore, left at rest for some time after the first heating, the spores germinate and are then easily killed by the subsequent heatings.

When water is to be sterilised under pressure it is heated for an hour in the autoclave at a pressure of 1 to 1½ atmosphere.

Yeast Water is an extract of yeast and is an extremely favourable culture medium for bacteria and yeast cells. Yeast water is prepared by boiling ½ kilogram of pressed yeast free from starch with 2 litres of distilled water for about half an hour; the liquid is filtered while yet warm and then the solution boiled for another half-hour and filtered, after which it is distributed in flasks and sterilised in a current of steam for about three-quarters of an hour. The yeast water thus obtained is however too concentrated for ordinary experiments. When, therefore, it is about to be used it is mixed with an equal quantity of sterile water, or as much as to make the mixture sherry-coloured, and then sterilised in flasks for three-quarters of an hour without pressure.

Meat Extract is prepared according to R. Koch in the following manner: 500 grams of meat free from fat, and 1,000 grams of distilled water, after being thoroughly stirred together, are left for twenty-four hours in an ice safe, or, during the winter, in a cold situation. The liquid is then expressed, boiled and strained, by which means the precipitated albuminous bodies are removed; 5 grams of sodium chloride and 10 grams of peptone are dissolved in every 1,000 grams of the meat extract, which is then neutralised with sodium carbonate. The liquid is now filtered at boiling temperature, sterilised for two hours and preserved in Pasteur flasks.

Fruit Syrups are most easily prepared from fresh fruit; after sufficient dilution with water they are sterilised for an hour in a current of steam.

If no fresh fruit is obtainable, dried fruit may be used as a substitute. For instance, a syrup may be prepared from dried apples in the following manner: 1 kilogram of dried apples, 5 litres of water and 20 grams of tartaric acid are allowed to stand for twenty-four hours; the mixture is then pressed, filtered and sterilised.

In the same way a grape juice can be prepared from raisins. But it is easier to use the concentrated grape juice obtainable as a commercial product recommended by Wortmann.[1] It is prepared in Sicily by evaporating the freshly prepared juice to about one quarter of its volume. It is viscous like syrup and contains about 65 per cent. of grape and fruit sugar, in addition to live yeast cells which, however, do not develop so long as the syrup remains concentrated. When it is about to be used it is diluted with 3 parts of water, clarified, if necessary, filtered and then sterilised in steam.

Solutions of Saccharose and Dextrose.—Other solutions in common use which might be mentioned are a 10 per cent. solution of saccharose in water, and a 10 per cent. dextrose solution in yeast water; both are sterilised for half an hour in steam.

Beer should be sterilised in the autoclave for a quarter of an hour under a pressure of 1 to 1½ atmosphere. It is not advisable to fill beer into flasks with rubber connections because, as mentioned before, the rubber tubing cannot stand the pressure. On boiling on the sand bath the whole of the alcohol would disappear; on the other hand, after sterilisation in the autoclave the beer contains half of the original quantity of alcohol. It has been shown that lager beer containing 5·65 per cent. of alcohol by volume retains 2·8 per cent. after sterilisation for a quarter of an hour under 1½ atmosphere pressure in Freudenreich flasks. If, therefore, sterilised beer is required with the whole quantity of alcohol the proper amount must be added either before or after sterilisation.

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[1] Concentrated grape juice is obtainable, *e.g.*, from Messrs. Favara and Sons, Mazzara del Vallo, Sicily.

Discontinuous Sterilisation.—Those substances which, from any cause, cannot withstand boiling may often be sterilised by a discontinuous process, the media being subjected to a temperature of 56-58° C. for two to four hours every day for a week.

Sterilisation by Filtration.—We have hitherto described the sterilisation of nutrient solutions by heat alone. In those exceptional cases in which this method cannot be employed, filtration through special filters is substituted. The best known of these are Chamberland's and Berkefeld's filters. In the first of these, filtration takes place through a tube of biscuit ware, the liquid being passed through by pressure or suction. The pores of the tube, however, are quickly clogged, rendering a frequent cleaning and sterilising necessary, the bacteria otherwise spreading through the filter. In the last-named filter the filtering medium consists of kieselguhr.

It is advisable to have a stock of the various culture liquids in concentrated form, as they do not then occupy so much room.

2.—*Solid Culture Media.*

Gelatine and Agar-Agar. — In the preparation of nutrient gelatine care must be taken not to make the heating too long or too strong, as the gelatine thereby loses its power of setting and is thus rendered useless. The procedure is as follows: The quantity of gelatine is weighed and placed in the boiling liquid, which has also been weighed, and diluted with the necessary quantity of water to prevent over-concentration. Heating is carried out in a dish on a sand bath, as gelatine solution is very easily burned; it is therefore advisable to keep stirring the mixture as long as it is over the flame. As soon as the gelatine has dissolved, the solution is removed from the source of heat and cooled to about 50° C., when a small amount of fresh albumen[1] is added; the latter is first beaten up with a little water, this being most readily done by shaking up violently in an ordinary medicine bottle. The white from one egg is sufficient for two litres of liquid. The gelatine solution is thereupon mixed well with the white of egg solution, the whole then put on the sand bath and cautiously heated to boiling without stirring. This coagulates the white of egg in a few minutes, and it separates out in large flocks which aggregate all the suspended matter and impurities present in the gelatine. The whole is now weighed to ascertain if the weight has remained the same: if the weight is too small, sterile water is added; otherwise the liquid must be carefully evaporated at a gentle heat until the proper weight is arrived at. The gelatine solution is strained through a flannel while it is still warm, the flannel being stretched on a wooden frame. In many cases a perfectly clear gelatine is not required, and it is then unnecessary to filter it. If on the contrary perfectly clear gelatine is required, it is filtered boiling hot through a paper filter provided with a toughened point. This filter is placed in a glass funnel fitted in a copper funnel (see Fig. 44). The latter is double walled and provided with a closed side tube. There is an opening on the upper edge of the funnel which allows of the space between the walls being filled with warm water. Under the side tube a gas flame is placed which keeps the water boiling during filtration; the gelatine is thus prevented from setting.

Smaller quantities of nutrient gelatine may be prepared with least trouble on the water bath.

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[1] According to Rich. Meissner the use of dry albumen is not to be ecommended, as it has been shown that when it is used for clearing the gelatine, organisms sown on the latter are hindered in their development. This probably arises from the formation of secondary products (ptomaines ?) during the drying.

Pasteur flasks are best for preserving nutrient gelatine as they do not allow any drying up. The strained or filtered gelatine while still hot is poured into a flask, previously sterilised, and boiled for five minutes on the sand bath. A smaller amount may be stored in different small flasks according to the purpose for which it is intended. As regards preservation of nutrient gelatine to be used for ordinary plate culture, it is advisable to use the globular flask represented in Fig. 36, which is charged with about 15 c.c. of gelatine. The gelatine is here also boiled for five minutes on the sand bath, after the flask has been plugged with cotton wool and covered with a double layer of filter paper. In case the flask is to remain for a long time, it is of advantage to use a rubber cap in place of the filter paper. These flasks are to be used in preference to test tubes or Freudenreich flasks, because the liquefied gelatine seeded with a culture can be better shaken up in the former than in the latter, it being above all desirable when making

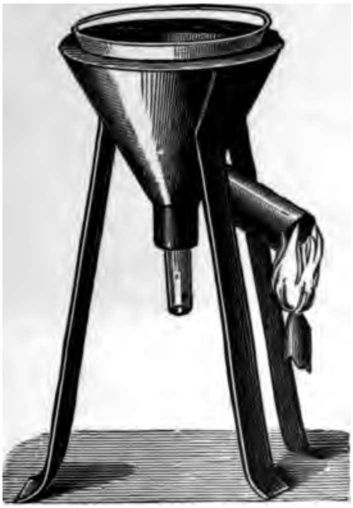

FIG. 44.—Hot Water Filter.

ordinary plate cultures to distribute the germs as much as possible. If the gelatine is to be applied for surface plate cultures, test tubes or Freudenreich flasks may be used in which the culture gelatine is only liquefied without being mixed with the germs; but globular flasks are also preferable in this case.

In Freudenreich flasks, gelatine is sterilised for a quarter of an hour in steam. After the sterilisation is finished, the flasks are placed in an oblique position until the gelatine has set. When these flasks are to contain their gelatine for a long time, the cap tube ought to be closed with wax or with the S-shaped tube mentioned previously (Fig. 33), to prevent the gelatine drying up. Gelatine ought in all cases to stand for some time before use, partly to see if it is sterile, and partly because it can then resist higher temperatures better without melting. With regard to the latter property, different kinds of gelatine behave differently.

Hueppe recommends the discontinuous method of sterilising gelatine, this consisting of subjecting it daily, for four to five days, to a boiling heat for five minutes.

Wort gelatine, yeast water gelatine, fruit syrup gelatine, etc., are prepared with a content of 7 to 10 per cent. of gelatine, that is, as much as will enable them to stand 25° C. without melting. Meat extract peptone gelatine is, on the other hand, always prepared with at least 10 per cent. of gelatine. Ten grams of gelatine are dissolved in 100 grams of meat extract in the usual way; sometimes it will be necessary after adding the gelatine to neutralise with sodium carbonate, for the gelatine, as is known, gives an acid reaction, and many bacteria do not thrive even on a feebly acid medium. Meat extract peptone gelatine is always sterilised by the discontinuous process.

Nutrient agar-agar is prepared in a similar way to the gelatine; but it must be cut into very small pieces before being put into the boiling liquid, and has to be boiled for some time before it dissolves. The proportions in this case are 100 grams of liquid to 2 grams of agar-agar. Filtering is, as a rule, avoided, as it is only accomplished with difficulty, agar-agar requiring a higher temperature to keep it liquid. Hence, white of egg is not used for clearing. If it is desired to remove coarser suspended matter, the solution can be strained through linen.

If, however, the agar-agar has to be filtered, Giesenhagen recommends the following method. To accelerate the agar filtration he employs filtration in steam, and distributes the substance among several filters working simultaneously. Small tin funnels with turned down edges are used for filtering, and these are provided with flat enamelled covers with projecting edges. Three funnels are placed in rings round the stem of a special stand arranged over Erlenmeyer flasks of appropriate size (each of the three filter stands is 16·5 cm. high and 8 cm. wide). The wadding plug for each flask is fixed in the meshes of the wire stand. Two, or in high steam chambers even three, such sets of filters (*i.e.*, 6 to 9 filters) can be arranged for steaming. The filtering is done through two folded filters. Nutrient agar-agar is employed for cultures at high temperatures, gelatine being unsuitable as it becomes liquid.

Mixtures of agar-agar with gelatine are prepared in the proportions of 100 grams of culture medium, 1 or 2 grams agar-agar, and 4 or 3 grams of gelatine. The addition of gelatine prevents the separation of water which always takes place when pure agar-agar is used.

Litmus gelatine is sometimes used as a reagent for detecting the formation of acid during the growth of a micro-organism. It is prepared according to Hueppe in the following way: An aqueous solution of the colouring matter is made up, sterilised and allowed to cool. The gelatine is liquefied at 30° C. (the agar-agar at 40°), and is then mixed with the equally hot litmus solution. The latter should only be so strong that its action as a reagent is just distinguishable.

Other Solid Media.—Bread might be mentioned as one of those solid media which are sometimes employed. It is made into a paste with a little water and sterilised by steam; rice may be sterilised in the same way. Manure (with or without addition of water) is sterilised several times with an interval of one or two days. These substrata are employed, *e.g.*, in cultivating moulds. Potatoes are frequently used for the culture of bacteria. The potatoes are cleaned well with a brush and laid for some minutes in a 10 per cent. solution of sublimate and afterwards in a 0·1 per cent. sublimate solution for half an hour to fifteen hours. Finally they are well washed with water and then sterilised for two hours in steam and cut up.

III.—METHODS.

1.—*Microscopical Investigation of Micro-Organisms.*

Preparation Making.—A preparation of a micro-organism is made as a rule by putting it in a drop of liquid or in Canada balsam, etc., on a glass slip and laying a cover glass on it. Water is often used (best when distilled and sterile so as to exclude outside organisms from the preparation); a little of the growth to be investigated is taken out with a platinum wire or similar instrument and stirred in the water. If it is in a culture liquid, a sample can be taken out with a small glass rod and placed direct on the glass slip without addition of water. The needles and rods used for taking out samples must of course be sterilised beforehand, when the cultures are to be preserved pure; all the usual precautions must be observed. When the cover glass is laid on the drop the enclosing of air bubbles in the liquid is to be avoided. These can, however, when formed, be expelled by a cautious tapping on the cover glass. Yeast cells and moulds are usually examined in the unstained condition.

Water-mounted preparations can be kept for some time if the cover glass is sealed round the edge to the glass slip so that no evaporation can take place. A very suitable medium for this purpose is a solution of common sealing wax in spirit, or a melted mixture of vaseline and beeswax. If it is wished to make really durable preparations, Hantsch's solution (see p. 92) is added drop by drop to an ordinary preparation in water so that after some time the only liquid remaining in the preparation is glycerine, the alcohol and water having evaporated. The gradual addition is necessary so that the form of the cells may not be altered too much by the water-absorbing property of glycerine. Afterwards the edge of the cover glass is sealed either with the above-mentioned sealing wax solution or with asphalt lac. This method is especially suitable for preparations of yeast cells and moulds. For the preparation of stained bacteria specimens see below. If the latter are to be made permanent they are mostly mounted in Canada balsam. In permanent specimens the cells always lose their natural form to some extent.

Removal of Grease from Cover Glasses.—In preparing a microscopical specimen which is to be fixed, hardened and stained, it is necessary to use perfectly clean cover glasses. To obtain these it is not sufficient to clean them in the ordinary way, but means must be employed to remove the thin layer of grease which always adheres to the glass. The cover glass is first laid in some strong mineral acid (hydrochloric or sulphuric), then washed with water and boiled in a soda solution; it is again washed with distilled water, dried, washed in absolute alcohol and again dried.

Fixing and Staining of Yeast Cells.—After the cover glass is carefully cleaned in this manner a drop of the culture is spread over it in as thin a layer as possible, and the cover glass left under a glass bell until the drop has completely dried up. If the culture is present in a solid substratum, a little of it is distributed in a water drop and the mixture spread on the cover glass. As regards the staining of a yeast cell preparation, *e.g.*, with an aniline dye, the preparation, thoroughly dried in air by the above method, is taken up by means of a pair of forceps with the prepared surface upwards and drawn through a small gas flame three times with uniform speed describing a vertical circle with a diameter of about one-third of a metre, the three motions occupying about three seconds. The specimen is thus fixed and hardened. A little of the staining solution is now put on the cover glass, allowed to act for some minutes and then washed off with distilled water. The clean side of the cover glass is next dried with filter paper, and the specimen is then ready for examination.

The distinguishing of dead cells from living ones has been assiduously carried on in most brewery laboratories since the microscope came into general use. But the value of the indications given by the reagents employed for this purpose has been very much overestimated. The question seems to deserve proper investigation. According to Wehmer, a half per cent. methylene blue solution will stain the dead cells indigo blue, while the living cells remain colourless.

Staining of Yeast Spores. — Ziehl's carbol fuchsine solution (see page 92) is used for colouring the spores of yeast cells. As soon as the preparation has been fixed in the above-described manner, it is laid in a small crucible or watch glass with carbol fuchsine, heated for a short time to boiling, and then washed with water, afterwards with dilute acid (5 per cent.), and then again with water. The spores are then usually coloured red; the rest is colourless. Sometimes other coloured bodies appear besides spores, and it may also happen that some single spores remain colourless.

Staining of the Yeast Cell Nucleus.—The detection of the cell nucleus is no easy matter. Janssens and Leblanc recommend a modification of Moeller's method, *viz.*, the following: A few drops of a solution of iodine in potassium iodide (1 part potassium iodide, 100 parts of water, iodine to saturation) are placed on a glass slip; and a little of the yeast in question is stirred in. A drop of the mixture is then spread on a well-cleaned cover glass. Immediately after the mixture has dried, the cover glass is put into the iodine solution and allowed to lie for twenty-four hours. The specimen is now hardened; the cover glass is taken out of the iodine solution, placed first in water, then in 33 per cent. alcohol, next in 80 per cent., and, finally, in 95 per cent. alcohol. Before proceeding to stain, the yellow colour of the cells must be completely removed. In case this is not effected by the 80 per cent. alcohol, an aqueous solution of potassium iodide (1 to 3 per cent.), or ether may be used. The specimen ought to lie at least forty-eight hours in the 95 per cent. alcohol; a longer soaking is not harmful, but is unnecessary. Carbol fuchsine is used for staining, the cover glass being warmed in a little of this liquid contained in a watch glass. The cover glass is then washed several times with water, and, finally, with very dilute sulphuric acid.

Heidenhain's method may also be adopted, the procedure being as follows: The fixed specimen is laid for four hours in a solution of 2·5 grams of iron alum in 100 c.c. of distilled water; it is then placed for twelve to eighteen hours in a solution of 0·5 gram of haematoxylin in 100 c.c.

of distilled water. Lastly it is decolorised in the usual manner.

Fixing and Staining of Bacteria.—A preparation of bacteria is stained and fixed in the same way as a yeast preparation. A drop of an alcoholic aniline dye solution is then allowed to act for some minutes, after which washing with distilled water takes place.

A special method of staining is described by Chr. Gram, which has found extensive application, chiefly because it is used as a method of diagnosing certain species of bacteria. According to this method the fixed specimen is stained from one to three minutes in a hot saturated solution of gentian violet in aniline water, and then immersed for one to three minutes or longer in a solution of iodine in potassium iodide. A precipitate is thus formed which is only deposited on the bacteria. The preparation is then washed with absolute alcohol until every trace of the colouring matter has been removed.

Staining of Bacteria Spores. — Bacteria spores are stained by boiling the fixed specimen for a long time in carbol fuchsine (in some cases for an hour, during which the evaporated liquid is constantly renewed); it is then washed in alcohol.

Aujeszky has recently communicated a simpler method for staining spores. A little of the culture containing the spores is spread on a cover glass, and while the smear is drying in the air, a half per cent. hydrochloric acid solution is warmed over a Bunsen flame in a porcelain dish until bubbles begin to appear. When this point is reached the Bunsen is removed, and the cover glass, now dried, but not fixed, is laid for three to four minutes in the liquid. The preparation is afterwards washed with water, dried, fixed and treated with Ziehl's carbol fuchsine, then held in forceps over the Bunsen flame and heated until it fumes. As soon as the staining solution begins to fume, the preparation is drawn out of the flame for some seconds, this heating being twice repeated. The preparation is then allowed to cool one to two minutes more, after which decolorising with 4 to 5 per cent. sulphuric acid follows. The latter process should not be carried too far in case the spores again become colourless.

Finally, we shall describe the method given by Alex. Klein, who found that spores easily become stained without any previous treatment if the dye is allowed to act on them in the moist state. The process is the following: Preparation of an emulsion of the spore-containing material in 0·7 per cent. salt solution (in a watch glass) and addition of an equal quantity of filtered carbol fuchsine solution, then gentle heating, steam being given off at the surface for six minutes, dust being kept off by covering with a second watch glass. The preparations are then spread out and allowed to dry in air and fixed by passing twice through the flame. Decolorising is effected by 1 per cent. sulphuric acid acting for one to two seconds, and lastly the preparation is washed with water.

Staining of Flagella according to Löffler.—Staining assumes a special significance when the question is one of detecting the motile organs, the flagella, of bacteria. There are several ways of doing this, one of the most used being that described by Löffler. After the preparation has been carefully fixed it is treated with a mordant, which consists of 2 parts of a 20 per cent. solution of tannin, some drops of an aqueous saturated solution of ferrous sulphate and 1 part of logwood extract (1 to 8).

Some drops of this mixture are placed on the cover glass and warmed directly over the flame until steam begins to form. Afterwards the cover glass is washed with water and stained with carbol fuchsine or with Löffler's solution, of which several drops are filtered warm on to the cov glass. This staining solution consists of 100 c.c. of saturat aniline water, 1 c.c. of a 1 per cent. solution of soda an 4 to 5 grams of gentian violet, fuchsine or methylene blue Washing with water takes place after staining.

Reagents.—The reagents used most frequently in our microscopical investigations are the following:—

Absolute alcohol.

Concentrated spirit.

Ether.

Chloroform.

Dilute soda solution (1 to 3 per cent.).

Dilute sulphuric acid (5 to 10 per cent.).

Dilute nitric acid (5 to 10 per cent.).

Perosmic acid (0·1 to 1·0 per cent. aqueous solution, kept in a brown or black bottle in a dark place, *e.g.*, in a tightly-closing cardboard box).

Iodine-potassium iodide solution (2 parts of potassium iodide, 300 parts of water, 1 part of iodine).

Tincture of iodine (a saturated solution of iodine in strong alcohol).

Iodine-zinc chloride solution.

Hantsch's solution (3 parts of 90 per cent. alcohol, 2 parts of water, 1 part of glycerine).

Carbol fuchsine (1 part of fuchsine, 5 parts of crystallised carbolic acid, 10 parts of alcohol, 100 parts of distilled water).

Tincture of alcanna (alcoholic extract of the alcanna root).

Development in Moist Chambers.—If it is desired to study under the microscope the development of a micro-organism, the moist chambers described on pages 68 to 70 are used. Böttcher's chamber (Fig. 42) is best suited for the cultivation of an organism which requires plenty of air in order to grow well. The micro-organism to be examined is seeded either in a hanging drop of culture solution or in a thin layer of nutrient gelatine on the under side of a suitable cover glass. One or two drops of water are placed on the bottom of the chamber and the cover glass stuck to the ring with vaseline. The cover glass can be still better fastened if, in addition, the edge is painted with a melted mixture of wax and vaseline. In examining organisms requiring a large quantity of air the cover glass may be laid on so as to leave a small opening, or the chambers are provided with special air tubes. The chamber should then be placed under a moist bell jar. Hollowed glass slips can be used in the same way.

Ranvier chambers (Fig. 41) may be used with the same facility for liquid, as the Böttcher chamber for solid, media. In using a Ranvier chamber the quantity of nutrient solution must not be so great as to run into the groove when the cover glass is fixed down; a drop of water is placed in the groove when necessary. In this case also the cover glass can be adjusted so as to leave the groove in communication with the outer air, and the chamber is then, like the former, placed in a moist glass enclosure.

2.—*Experiments with Various Flasks. Inoculation of Liquid and Solid Culture Media.*

The Manipulation of Nutrient Liquids requires considerable practice. We will give in the following a description of the various devices so far as this is practicable. Let us suppose that it is required to transfer a liquid, with or without a growth in it, from one Pasteur flask to another, without infecting the liquid; we should proceed in the following manner. The table on which we are working is first moistened with the mixture of spirit and water previously described, the gas burner and tubing being also washed. This is a precaution which is to be observed in all such experiments. Coat sleeves should fit tightly to the wrist and for this purpose rubber bands are used, or, still better, a linen overcoat with tightly fitting arms is worn. Above all it is desirable that no dust should be introduced. The gas flame (the tubing should be connected to the left) is placed directly in front, and between it and the operator the tinned copper vessel already mentioned which is sterilised in the gas flame. The flask from which the solution is to be poured is placed on the left, the other on the right, and both flasks as near the gas flame as possible. The copper vessel is placed between the two. The arrangement may be seen from the accompanying sketch in Fig. 45. K_1 is the flask from which we wish to pour solution into K_2. Both flasks as well as their supports are then carefully sterilised on the surface by means of the gas flame; if they contain cultures this must be done with great care, so that the organisms are not killed by the heat. The bent tube is now heated to redness, beginning at the bulb in the middle of the tube and then going downwards to the end. The asbestos plug is then taken out. If it is wished to transfer an average sample the flask is shaken up, the lower bend of the tube being held in the flame during the shaking up, so that the air passing in may be sterilised. Care must be taken that the point of the tube is kept out of the flame, otherwise gas would be sucked into the flask with danger of an explosion. The gas flame is put in its place again and the glass plug of flask K_2 loosened, without being taken out (if this is done it must be in the flame), so that it remains only with its end fitting loosely in the rubber tube. The Bunsen burner is now regulated so as to give a luminous flame because the

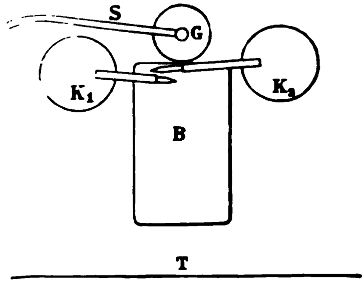

FIG. 45.—Arrangement of two Pasteur Flasks (viewed from above). S, the gas tube; G, the burner; K_1 and K_2, the flasks; B, the copper dish; T, the edge of the work table.

latter is not so hot as the non-luminous; the flask, K_1, is then taken in the left hand, the body of the flask being held; and the rubber of the side tube is loosened with the right hand. The rubber is then quickly taken off in the flame and laid in the copper dish, the opening of the side tube remaining in the flame, while the tubing of the flask, K_2, is squeezed with the right hand so that its glass plug falls into the copper dish. For the moment the situation is as follows: The left hand holds K_1 so that the opening of its side tube is in the flame and the right hand is occupied in pinching the rubber tube of K_2; the side tube of K_1 is now fitted into the rubber of K_2 in the flame. This should all be done so quickly that neither the tube nor the rubber is over-heated.

The two flasks are now in communication with each other and we leave them in this position without transferring any of the liquid so that the heated tube of K_1 may cool down. This takes place in quite a short time, whereupon the burner, the flame of which is again made non-luminous, is held in the left hand and the bent side tube of K_1 made red hot, after which K_1 is tilted so that the solution can run from it into K_2, the bent tube of K_1 being heated as long as the solution is being poured so that the air passing in may be sterilised. When the required quantity of liquid has been passed over, the Bunsen burner is put back in its place. The glass plug of K_2 is now taken up with the right hand and sterilised in the flame, which is then made luminous; the glass plug being held between the first and second fingers of the right hand, the rubber of K_2 is held between the thumb and first finger of the same hand, and the side tube of K_1 disconnected, being placed quickly in the flame; the glass stopper is immediately inserted in the rubber of K_2 and the rubber of K_1 carrying its glass stopper is at once lifted out of the copper dish with the right hand

and placed on the side tube of K_1 the opening of which i still in the flame.

This procedure may perhaps seem somewhat complicated after reading through the above description, but if it has been tried in practice it will be found to offer no special difficulty but only requires practice. The very first exercises can be performed with flasks which contain ordinary water; then flasks may be used containing sterile wort, allowing them to stand between experiments in order to see if infection has been avoided. After some practice flasks should be used which contain sterilised yeast water or meat extract in order to see if these remain sterile with constant use; this is much more difficult, as most bacteria develop readily in these liquids, which does not happen in wort. If it is seen that these flasks remain sterile after they have been worked with for several days, it may be then assumed that the necessary experience has been acquired.

A similar method to the above is adopted in using Hansen flasks which are also provided with side tubes. But the air entering does not require to be heated after passing through the cotton wool in the cap, since the latter acts as a filter.

Sterilised pipettes have to be used in experimenting with Freudenreich, Chamberland and Erlenmeyer flasks. These flasks also are of course always sterilised beforehand with the flame. This has to be done also when a part of the contents of a Pasteur flask is to be passed into one of the above-named flasks. If only small quantities of liquid are being used, sterilised glass rods or metal wires may replace the pipettes. These experiments should be performed in the sterile cupboard or in the sterile room, and it is necessary to work very quickly. Exercises ought also to be performed with the pipettes as with the Pasteur flasks, and the operator ought not to consider himself efficient until he can keep flasks containing yeast water or meat extracts sterile after repeated manipulations.

Experiments with Solid Culture Media.—If a portion of a growth is to be inoculated from a solid culture medium into a flask containing nutrient solution or on to new solid medium, then metal wires, such as platinum, or glass rods are used. Before use they are sterilised in the flame and placed in a flamed tin box, which is covered with a glass plate likewise flamed. They are left in the box until quite cool. This is very important, as one might otherwise easily kill the growth to be introduced. Immediately before use they are once again drawn quickly through the flame. When glass rods are to be used it will be better in many cases to take one drawn out into a long thin point, especially when small specks of growth (*e.g.*, from a moist chamber) or a fine mycelium, which clings easily to inoculation needles when these are used, has to be introduced, as the point of the rod can be broken off and left in the infected culture liquid. The infection is thus performed more surely and more quickly than when a metal wire has to be rubbed against the sides of the flask in order to leave particles of growth in the liquid. Sometimes small pieces of platinum wire are used for the same purpose, being manipulated by a pair of forceps.

Infection of a solid culture medium takes place either on or below its surface. A surface culture is laid on most easily as a streak made with a metal wire or glass rod; if the culture to be introduced is contained in a liquid, a pipette may be used, by means of which we then sow a drop on the surface of the solid culture medium; or we may use a metal loop. On the other hand, if the culture is to be sown in the substratum, this may be performed by aid of a metal wire (an inoculation needle), which with the adherent growth is thrust into the medium; a so-called "stab" culture is thus obtained. Development of the organism then takes place on and below the surface. Cultures in the body of the substratum may also be obtained by mixing the growth with liquefied nutrient gelatine.

Plate cultures, which are employed in the production of pure cultures, are described on page 103.

Cultures of Anaërobic Organisms.—If it is wished to start a culture of an anaërobic organism on a solid medium, this can be done by covering the medium with a plate of mica (Koch), which is pressed on, the medium having a perfectly level surface. A glass plate can be used in place of the mica, or melted gelatine is poured on to the infected medium (Hesse). Of other methods that of H. Buchner may be mentioned. According to the latter the organism under examination is placed in a small test tube with nutrient gelatine, and this put into a second larger test tube in which there is an alkaline solution of pyrogallic acid (1 vol. of an almost saturated solution of pyrogallic acid mixed with 10 vols. of potash solution [1 + 1]); the outer test tube is kept well closed with a tight plug. The alkaline solution of pyrogallic acid absorbs all the oxygen so that the culture in the inner open test-tube grows in an oxygen-free atmosphere.

The process may also be carried out by displacing the atmospheric air by an indifferent gas, *e.g.*, by hydrogen. The method described by Fränkel consists in the use of ordinary wide test tubes fitted with double-bored bungs through which two glass tubes pass, one reaching almost to the bottom, the other ending just below the bung. This culture tube after being filled with the gelatine is sterilised, inoculated, and then has a current of hydrogen passed through it. After all the air has been driven out, the glass tubes are closed by fusing.

Suppression of Bacteria in Yeast Growths.—If it is required to encourage the development of a bacteria-infected culture of an alcoholic yeast, the method usually adopted is to cultivate the impure growth in an acid nutrient medium. Nearly all bacteria are killed in this way, or are to a great extent hindered in their development, so that the alcoholic yeasts in the mixture preponderate. But it is not always possible to use such an acid medium, as the organism to be cultivated may also be influenced adversely. In such a case the cultures can be exposed to the action of light, as bacteria can withstand the action of light only to a small degree. This was proved several years ago by Downes and Blunt. Experiments in this direction have also been made at the Carlsberg laboratory, and have shown that spore cultures of saccharomycetes on gypsum blocks can be kept free from bacteria if exposed to light.

3.—*Preparation of Pure Cultures.*

The methods for preparing pure cultures described in the following pages are only those of practical importance and application.

PURE CULTURES FOR INVESTIGATIONS IN MORPHOLOGY AND DEVELOPMENT.—The preparation of pure cultures for investigating morphology and development was begun very early, the development of the single cell being observed under the microscope. This was perhaps first done by Ehrenberg (1821); later, Mitscherlich studied the budding of the yeast cell in the same manner. The process of microscopical examination as a means of studying morphology and development was largely employed in investigating fungi belonging to the most widely separated divisions of the system. The technique was brought to a high degree of perfection, especially by Brefeld (1875).

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Brefeld's Glass Slip Cultures.—The essential point in this method is the direct microscopical observation of all stages of development. Brefeld takes a fruit carrier with spores, or a small mass of spores, from the mould in question, *e.g.*, a *Mucor*, with the aid of a fine needle and distributes them in a drop of sterile water; he continues diluting until only one or two spores are present in any drop, and one of these drops is then placed on a glass slip. He then adds nutrient solution, the position of the mould spore is marked, and, to prevent evaporation, the glass slip culture is kept under a moist bell-jar when not under observation. If the investigation is prolonged, the culture medium is replaced by gelatine to prevent evaporation. These Brefeld glass slip cultures are quite open, which is of some importance in the investigation of the higher fungi as they have then sufficient room for growing; the culture, however, is in this way exposed to infection from the air. In order to mitigate this danger, a paper shield is placed on the microscope. But even when foreign germs, *e.g.*, a *Penicillium* spore, find their way into the culture, it is of little importance; no error can be made, as the culture is under microscopical observation the whole time. This technique is excellent in its own sphere.

Brefeld in his investigations on *Bacillus subtilis* used moist chambers, *viz.*, those of v. Recklinghausen. The latter are only suitable for morphological work and have not found a place in this book, all the more so as they have been recently replaced by a better design. (See the moist chambers mentioned on page 68.)

PURE MASS CULTURES.—If the pure culture, on the contrary, is to lead to an absolutely pure mass culture, other considerations arise than when the former is designed only for morphological and developmental purposes. In the latter case it is of no importance if a foreign organism is present along with those being cultivated, for the whole investigation is carried out on the stage of the microscope under continued observation. It is quite otherwise when the physiological experiment is carried on with flasks containing mass cultures; another technique is here necessary.

These pure culture methods are divided into two groups, one comprising those based on the principle of dilution, whilst in the second the physiological behaviour of the species forms the basis. It is only by use of the dilution method, and that in its most developed form, that an undoubted pure culture is obtained. Such a culture is prepared by sowing out a single cell in a sterile culture medium and by further cultivation in such a way that no foreign organisms are able to force their way in.

I. *The Dilution Methods.*—Dilution methods may be again divided into two groups, according as the dilution takes place in nutrient liquids or solids. In the dilution of nutrient liquids the method is to count the number of germs in a certain unit of volume, and then to dilute with a calculated quantity of the liquid until there is one cell per unit of volume. This method was used by Lister (1878, pure cultures of a lactic acid bacterium), Nägeli, Fitz and Hansen (1882). The method was brought to its greatest perfection by Hansen.

Hansen's Dilution Method.—The dilution method as used by Hansen's predecessors was quite uncertain. It was never really known whether those flasks in which a growth was developing contained a pure culture or not, *i.e.*, whether the seeding consisted of one or several cells. The counting method in use was not exact; but even with exact counting the seeding might consist, in certain flasks, of more than one cell. Therefore, Hansen added to the method two elements, by means of which it gained in certainty, *viz.:* (1) an indication to decide whether the infected flask has received one cell or several; and (2) an aid to the exact counting of the cells. Hansen applied the following method: The yeast (Hansen's method can only be used for the heavier cells, and thus not for bacteria) was mixed with sterile water, in which the cells were distributed by continued and violent shaking. A drop was taken out, and the number of cells in a drop determined by means of the squared cover glass (Fig. 4) described on page 30. The squares of the cover glass rendered it possible to perform an exact counting. The mixture was thereafter diluted to such an extent (by calculation) that there was, at the most, one cell in every two drops. A drop of the mixture was then sown in each of a series of flasks containing wort, after which the flasks were shaken up vigorously for some time in order to separate the cells, in case it should happen that more than one had been introduced; they were next set away and left undisturbed, so that the cells could sink to the bottom. Those flasks in which only a single yeast spot (colony) formed had thus received only one cell, and contained an absolutely pure culture. This, which later formed the starting point for Koch's plate cultures in nutrient gelatine, constituted, in conjunction with the direct counting of the cells present in the drops, the exactness of the method. By means of this method Hansen prepared the first pure cultures of his *Saccharomyces* species.

In order to control the exactness of his method he instituted special experiments in which he mixed two species of yeast which could easily and with certainty be distinguished from one another under the microscope; the mixture consisted of *Saccharomyces cerevisiae* or *Saccharomyces Pastorianus* with *Saccharomyces apiculatus*. The results demonstrated the exactness of the method; where only one yeast spot had formed there was but a single species in the flask.

The direct sowing out of a single cell can, of course, be done also with the aid of the squared cover glass. Since the easier gelatine method described below has been placed at our disposal, the above dilution method is only used in isolated cases. This happens, *e.g.*, when it is intended to prepare a pure culture from very much emaciated cells, or when the number of living cells is to be determined in a growth of which most of the individuals have died. Emaciated cells, as mentioned above, do not develop at all in wort gelatine, but do so in wort.

Dilution in and on Solid Substrata.—When a solid medium is used for dilution a plate culture is prepared; Schröter was the first to introduce them (1872). He observed, on slices of potato exposed to air, a gradual formation of spots of different shape and colour, produced by the bacteria in the air. On investigation of these spots he found that each, as a rule, contained only one species.

R. Koch used (1881) gelatine mixed with a nutrient solution for the preparation of pure cultures. He distributed the germs in the solidified gelatine by inoculation by streaks. The number of germs introduced into the gelatine becomes less and less for every additional streak, so that the colonies in the last streaks are isolated ones. But it does not follow from this that they contain pure cultures.

Koch's Plate Culture.—Koch in the year 1883 replaced his streak method by his plate cultures and obtained by means of the latter a more complete separation of the cells. This method consists in distributing the germs in liquefied gelatine, the mixture being poured out on a horizontal glass plate, which is set under a moist bell jar and protected from outside germs. The cells are fixed by the solidification of the gelatine, and colonies develop, which, in the course of a few days, become visible to the naked eye. The number of cells in the gelatine should not be too large; there must be sufficient room for the development of the colonies. Instead of pouring out the gelatine on a glass plate, Petri dishes (see Fig. 37, p. 64) are now commonly used for this purpose. As, however, the dish does not afford complete security against infection, special precautions must be used to ensure safety. The procedure is as follows: A little of the growth to be separated, usually a mixture of various organisms, is placed in sterile water, for example in a Freudenreich flask or better in an ordinary globular flask (see Fig. 36, p. 63), in which the cells in the water can be distributed by shaking; it is here necessary to separate and distribute the cells in the water as well as possible. Some nutrient gelatine contained in a similar flask is melted on a water bath heated to about 35° C. When the culture is liquefied and the water mixture sufficiently shaken up, a small quantity of the latter is placed in the liquefied gelatine, and this mixture now well shaken up, care being taken, however, that no air bubbles are formed in the gelatine. The flask containing the gelatine is provided with a plug of wadding; the mouth of this flask is put quickly into the flame and simultaneously the cotton wool plug removed by forceps and again replaced. After cooling, the plug is again taken out and the liquefied gelatine mixture poured quickly into a Petri dish which must be quickly closed with its cover. The dish is then allowed to remain undisturbed, until the gelatine is quite firm; then the culture is brought up to the desired temperature. It is not advisable to place the dish with the liquid gelatine on ice in order to cool it more quickly, as the air then passes in too quickly, and outside germs easily find their way in. The simultaneous preparation of several plate cultures with varying additions of the growth of organisms is to be recommended. Beginners usually get far too many cells in each plate. It is of assistance in the investigation of the water mixture if a counting is done under the microscope. The preparation of plate cultures should always be carried out in the sterile chamber.

This pure culture method is specially adapted, as mentioned before, for the separation of the various elements of mixed cultures. It has, however, its disadvantages as well. There is, for instance, no security that the developed colonies arise from single cells.

Hansen tested the Koch method in the same way as his own pure culture method, following the same procedure, and using the same yeast mixtures as were mentioned on page 102, and prepared some plate cultures by its means. The result was that 1·5 per cent. of the colonies were formed of both species, while the remaining colonies were pure cultures, either of the one or the other. Holm found that the source of error is usually larger; he carried out a thorough research, the result of which was, as regards the yeast cells, that, on an average, 100 colonies were formed from 108 cells. He found, further, that the cells are more difficult to separate from one another at the beginning of the fermentation than at the end. Therefore the error is smaller if the plate culture is made with cells in the latter stage. But, on the other hand, a large number (25·5 per cent.) of cells do not then develop on account of their weakened condition. This number is reduced to 4·5 per cent. if the cells are taken at the beginning of the fermentation. Wort gelatine as compared with wort is, on the whole, less favourable to development. If it is wished to separate an impure brewery yeast, so as to isolate the culture yeast, it should be noted that the wild yeast preponderates at the end of the fermentation.

Miquel carried out similar investigations with respect to bacteria; he found that 100 colonies were formed from 134 cells, thus giving a still more unfavourable result.

Surface Plate Cultures.—A modification of the plate culture is the so-called surface plate culture (W. Kruse). The process consists in pouring the melted gelatine (before infection) into a Petri dish and allowing it to become quite firm. After complete setting, a suitable quantity of the water mixture containing the organisms is placed on the gelatine and spread out carefully over the surface by means of a sterile platinum brush (see page 71). The result is that all the developed colonies can be easily taken out.

Hansen's Second Pure Culture Method. — In the same year, 1883, that Koch introduced his plate culture described above, Hansen worked out his second pure culture method for yeast cells. In doing so, he took advantage of the technically important step made by Koch, *viz.*, the use of culture gelatine, but he added a new element to the process in controlling the development of one cell into a colony under the microscope. Only by this means is it made absolutely certain that the single cell forms the starting point. On the other hand this method cannot be used for most types of bacteria, as they are too small to be observed singly in the gelatine.

The method is as follows: A suitable mixture is made from the yeast growth by means of sterile water, so that the number of cells after mixing a drop of the mixture into liquefied wort gelatine[1] is not too great (see below). This, however, requires practice. Some information may be obtained, as mentioned above, from the microscopical examination of a drop of the water mixture; the approximate number of cells in the drop is counted by aid of a squared cover glass;, it can then be calculated about how many drops of the water mixture are to be added to the gelatine, in order to have a convenient number of cells in one drop of the gelatine mixture. This number is 20 to 30 cells if a Böttcher chamber with a ring of 30 mm. diameter is used. But after some time it will be possible to prepare the proper mixture without counting or calculation.

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[1] At ordinary temperatures a 4 per cent. culture gelatine can be used. The colonies then develop in the course of seventy-two hours in such a way that they can be removed.

When a suitable mixture of yeast and sterile water has been thus prepared, several Böttcher chambers are sterilised in the flame and placed under a sterile bell jar or sterile beaker to protect them from dust; all these experiments are carried out in the sterile cupboard. A very small drop of sterile water is then placed on the bottom of each chamber and some vaseline melted in a small saucer over a flame. The edge of the ring of the Böttcher chamber is painted with the melted vaseline, the latter substance being used because, after solidifying, a completely homogeneous mass without air bubbles is obtained. The requisite cover glasses are then flamed and likewise placed under sterile bell jars or small beakers. Finally a flask with wort gelatine is placed on a water bath at 30 to 35° C. to liquefy the gelatine.

A little of the yeast water mixture is mixed with the proper quantity of liquefied wort gelatine in a globular flask, and after being shaken vigorously, the formation of air bubbles being avoided, a drop is taken out with a thin glass rod or a fine pipette and spread out in a thin layer on the cover glass. The latter is left under the sterile bell jar for some minutes until the gelatine has set. It is then placed with the gelatine layer downwards on one of the Böttcher chambers and pressed firmly round the edge so that the vaseline closes it completely. The edge is painted with a melted mixture of 2 parts of vaseline and 1 part of wax to prevent the cover glass slipping. The Böttcher chambers are often set up, as mentioned previously, in such a way that the cover glass is fixed to the loose ring with fish glue, and the ring is then fastened to the glass slip by means of vaseline or with a mixture of wax and vaseline.

We proceed now to investigate the chambers with not too great a magnification. Some practice is required in finding the cells: and it must not be forgotten to investigate the gelatine layer through its whole thickness, so that any cells deeply embedded may not escape notice. When a well-isolated cell is found, its position is marked; this is done either by means of the object marker of Klönne and Müller, by the use of squared cover glasses, or by using a stage with a scale or fixed mark (see pages 28, 30 and 33).

When we have by some means marked as many cells as is desired, and have convinced ourselves that there are no other cells in their immediate neighbourhood, the chambers are put away at the temperature of the room or at 25° C. If precautions are not taken, water drops are usually formed on the under side of the cover glass, especially at the edge of the gelatine and, what is worse, on the gelatine itself. To prevent the formation of these water drops, the chambers are placed under a moist bell jar which has previously been brought to a temperature a little higher than that in which the chambers are to be kept. It is advisable to examine the chambers after twenty-four hours in order to confirm the isolated positions of the marked cells.

When the colonies have become large enough, they are cautiously transferred to the nutrient liquid (*cf.* page 97) either by means of a piece of platinum wire held by a pair of forceps or by a very thin glass rod, the point of which can be easily broken off in the liquid to be infected.

In order to be able to use the object marker of Klönne and Müller, already mentioned, the objective is unscrewed and the point of the object marker coated with a dye solution. Since the chambers are often placed under a moist bell jar as described, a colour must be chosen which can withstand moist air and does not spread. Holm found that a suitable colour may be prepared from 0·25 part of fuchsine dissolved in 2·0 parts of aniline and mixed with 2·0 parts of a xylol solution of Canada balsam. A drop of this colour is spread in a thin layer on a small glass plate such as a glass slip. The point of the object marker is now pressed against the medium, so that the edge of the opening is distinctly coloured. Care must be taken that the colour film does not spread over the opening; if this happens it can be easily removed by blowing through the other end of the apparatus. The object marker is now screwed on to the tube of the microscope, and so adjusted that the point almost touches the cover glass of the moist chamber. The tube is then screwed downwards very cautiously for a small distance by means of the micrometer screw so that the point of the object marker touches the cover glass, with which it is allowed to remain in contact for about ten seconds, after which it is raised again. A red ring is thus stamped on the cover glass, inside of which is the isolated cell under observation. It is not advisable to fit the object marker on to a revolving nose-piece, for it often happens that the field of view of the objective and the opening of the object marker do not exactly coincide, and the cell therefore lies outside the coloured ring.

Sometimes it is a little difficult to mark distinctly on the cover glass. This is caused partly by the point of the object marker not being quite plane and partly by the coloured liquid not having the right consistency. Under such circumstances it is found that the best way is to allow the first layer of colour placed on the point of the object marker to dry on and then to apply a new layer. The first dry layer then acts as an elastic cushion. The use of this apparatus requires practice and, as may be inferred, a light hand to avoid breaking the cover glass.

Squared cover glasses, with or without numbers in the squares, may be used, as already mentioned, instead of the object marker. In the first case the squares can be made larger than in the latter. The chamber is set up as described above, the isolated cells being marked in the following manner: If there are numbers in the squares (see Fig. 6, page 30), the square with its number and the position of the cell with respect to this number is drawn on a piece of paper. A fixed point is thus obtained and it will be easy to find the cell again. If there are no numbers in the squares (Fig. 5, page 30), each square can be designated by means of two numbers, the one being the number of the horizontal row, the other the number of the vertical column which contains the square. For example 3, 4 means that square which lies in the third horizontal row and in the fourth vertical column. In this case there must, of course, be only one cell in each square.

Lindner's Droplet Culture. — The methods described above can, of course, be varied in several ways: one of these, for instance, is Lindner's droplet culture (1893). He diluted a wort culture until only one cell was found in every streak or dot which he made with a drawing pen on a cover glass. The cover glass was then turned over and fixed with vaseline on a hollow glass slip or on a Böttcher chamber and the preparation examined microscopically. Those droplets showing only one cell were marked with ink dots on the upper side of the cover glass. After a few days the growths have developed and those droplets which contain the growths originating in one cell are sucked up by means of a small piece of sterile filter paper; the latter is then placed on wort gelatine in a flask, and a drop of wort is added in order to accelerate development. Instead of taking up the drop on filter paper, a little gelatine may be added; the whole is then taken up on a platinum wire or similar instrument and introduced into the wort. Lindner accordingly begins with a liquid, then uses gelatine, and only after a growth has formed on the gelatine are the mass cultures prepared in flasks containing nutrient liquid.

Schönfeld's Method.—Of the dilution methods that of Schönfeld remains to be described. According to him a dilution is prepared by means of liquefied culture gelatine, and small spots are placed on a cover glass from this gelatine mixture by means of a drawing pen. Each spot ought, as far as possible, only to contain one cell. When this method is further considered it will be seen that the spots ought only to be so large that the whole of each may be in the field of view when using a medium magnification, and it will be found necessary to add a little more gelatine so that the small gelatine spots may not dry up and in order that a growth may take place at all. The method is, as may be seen from the foregoing, a combination of those of Hansen and Lindner.

II. *Physiological Methods.*—It often happens that the organism which is to be cultivated in a pure state is present in comparatively small numbers, and the above-mentioned dilution methods cannot then be applied. We must then resort to a physiological method. These methods, however, are by no means exact, and offer no certainty of obtaining a perfectly pure culture.

Fractionated Culture after Klebs and Others.—A method in general use among the older physiologists and bacteriologists was a combination of an imperfect dilution method with a physiological method. Klebs's method, the so-called fractionated culture, is an example of this, as it consists in inoculating new sterile culture liquid with the previous culture as soon as that has developed. In this way Klebs expected to obtain finally a pure culture of the species present in greatest numbers at the beginning. The preponderance of one species is therefore a condition for the use of the method; but that species which was in the majority to begin with may not survive best, so that it is questionable in the case where a pure culture is obtained whether it consists of the species looked for. The pure culture obtained will, at any rate, belong to that species which increases most strongly under the prevailing conditions: but this will not in every case be the species of which it is desired to obtain a pure culture.

Pasteur's Method.—Pasteur (1876) also employs this physiological principle. He gives some indications as to the possibilities of obtaining a pure culture, by making use of the various physiological properties of micro-organisms, but chiefly of the greater or less capacity of increasing in different culture media, or, if the culture liquid is an unfavourable one, of the greater or less resisting power. A struggle between the species is thus brought about, in which possibly the weaker is killed, but this is not certain; those species, too, which are equally strong will be able to live peaceably together, and on this account also it is uncertain whether a pure culture is obtained. This holds in general for the addition of chemical substances to the culture medium, in order to favour the development of one organism at the expense of the other. Pasteur's use of tartaric acid in the preparation of pure yeast may be instanced; it affords a distinct proof of the uncertainty to which such a method may lead. Since the properties of that species of which it is desired to make a pure culture are in most cases unknown, the method is also less attractive for the reason that it simply assumes that the properties of the species are known. The above holds also for the employment of certain temperatures for the same purpose. The physiological method is only important in so far that it is a preparatory one; single cell culture must be employed for the preparation of a genuine pure culture.

Pure Cultures of Bacteria.—In the foregoing we have had chiefly in view the pure culture of yeast cells; with regard to bacteria, the physiological method may be employed in general for a preparatory cultivation, otherwise Koch's plate culture is the most suitable means, since bacteria are too small to be recognised with certainty in an isolated state in gelatine or liquids. The plate culture is repeated several times, the starting point each time being from a colony in the previous plate culture. The probability of obtaining a pure culture increases with the number of plate cultures. Sometimes the appearance of a colony affords a means of determining whether a pure culture has been reached, as this is often characteristic of the single species; microscopical examination of the cells also assists, of course, in the elucidation.

Pure Cultures of Mould Fungi.—In order to prepare a pure culture of mould fungi, a single sporangium (*e.g.*, of *Mucor*) or the conidia of a single conidiophore (*e.g.*, of *Penicillium*) is touched with a sterile needle. The adhering sporangium spores or conidia are then inoculated into a nutrient medium such as wort or wort gelatine, or an additional step may be taken, the spores being distributed in water and a plate culture in wort gelatine prepared. In the foregoing, reference has been made to Brefeld's investigations on the development of mould fungi. In his treatises are to be found very valuable directions on this point.

4.—*Methods of Preservation.*

Hansen's Saccharose Method for the Preservation of Yeasts and Moulds.—It is of great importance in physiological fermentation laboratories to be able to keep pure cultures of the various micro-organisms in such a way that it is not necessary to make frequent additions of new culture medium in order to keep them living. Hansen has elaborated a method of preserving yeast fungi and many mould fungi, *viz.*, by storing in a 10 per cent. cane sugar solution. As regards yeasts the process is as follows: A strong young growth of the species of yeast to be preserved is cultivated for twenty-four hours in wort at 25° C. The top liquid is all poured off from the settled yeast and a small quantity of the latter placed in a 10 per cent. aqueous solution of saccharose, which is set away in a flask at not too high a temperature. Ordinary room temperature is the highest working temperature, and if the flask used is a Freudenreich or a Hansen flask, it must be kept in a dry place so that moulds may not grow through the tube of the cap. The evaporation of liquid in a Pasteur flask is quite insignificant; some cultures in saccharose have been kept in Pasteur flasks in the Carlsberg laboratory for more than twenty years, as previously mentioned, without showing any noteworthy evaporation of liquid. The evaporation from the other flasks is also tolerably small, if the cotton wool in the cap tube is not kept too loose, the tube being of the proper length and the cap fitting well. Flasks of the latter kind can also be kept for several years without a renewal of liquid being necessary, if they are well filled at the beginning. (*Cf.* pp. 61, 62.)

So far as the saccharomycetes are concerned the vitality appears to be almost unlimited if the preserving takes place in the above manner. Numerous cultures of these have been kept alive for more than twenty years. Death has seldom taken place, and then only in a few species. This method has thus given excellent results. It has been recommended that the pure cultures should be allowed to stand in the fermented wort, that is in beer, and preserved in this way. But it has been shown that there is then no certainty that the growths will be kept alive, the duration of life when such a method of preservation is used being subject to great variations. The same species—this holds for all saccharomycetes—lives in some cases in beer only for a few months, in other cases for several years. Beer is therefore of little utility as a preserving medium, since the cultures must be renewed every two or three months; it is, of course, almost impossible to do this in an ordinary laboratory.

It has been advanced against the saccharose preservation method that the saccharomycetes increase in it and form yeast rings and films, of which the cells vary in morphological and physiological characteristics from the original seeding, and give a progeny which inherit these new properties. On investigating the numerous old saccharose cultures of the Carlsberg laboratory, the author arrived at the result that yeast ring and film formation only take place in saccharose solutions when the seeding, and therefore also the increase, has been too great. According to Hansen's researches the increase from a scanty seeding in a saccharose solution is limited, but if a larger amount of yeast is seeded, a vigorous increase, as mentioned, takes place, accompanied by the formation of films and yeast rings; even when the yeast is washed beforehand this can occur, the stronger cells living at the expense of the weaker. Under these circumstances it is possible that numerous generations are cultivated under abnormal conditions and that thereby a variation may ensue. The author obtained proof that the above views are correct by instituting a comparative series of experiments with culture yeast (a German bottom yeast and *Sacch. cerevisiæ* I.) and wild yeast (*Sacch. Pastorianus* I.). In no single case was the smallest trace of a yeast ring or film observed, when the seeding amounted to a trace only, the liquid just becoming turbid; but the contrary took place in nearly all cases where 5 to 7 drops of bottom yeast, either washed or with adherent culture medium, were placed in Freudenreich flasks. Experiments with two of the Hansen non-sporulating varieties of *Sacch. cerevisiæ* I. and *Sacch. Pastorianus* I., which had been seeded in the same way, demonstrated that these forms which had lost the power of film formation simultaneously with the power of spore formation, naturally formed neither films nor yeast rings even when the seeding was considerable. But when the surface of the saccharose solution with the scanty seeding of the original forms as well as of its varieties was examined macroscopically, single cells were seen floating about. Now do these cells belong to the surface film? No, for, first, the macroscopic film and the yeast ring are wanting, and, secondly, these single surface cells are found in the non-sporulating varieties named, which form absolutely no film, as well as in the original forms. Thus there is no question here of a film formation, but only of differences in the specific gravity of the single cells; some of the cells are too light to sink to the bottom of the denser saccharose solution. There is therefore no danger of a yeast ring or film forming in the saccharose cultures, if the seeding has been performed in the manner described above.

A large proportion of the moulds are capable of preservation in a 10 per cent. saccharose solution. This holds, *e.g.*, for *Mucor*, *Aspergillus* and *Penicillium*, besides for other fungi, such as *Monilia*, *Oidium*, *Torula*, *Mycoderma*, *Dematium*, *Cladosporium*, etc. The life of these fungi also may, according to Hansen's researches, be prolonged for many years.

Preservation on Cotton Wool or Filter Paper (after Hansen).—When saccharomycetes are to be preserved for a shorter time, cotton wool or filter paper can, according to Hansen, be used, especially when a pure culture has to be sent away. A small quantity of freshly cultivated yeast sediment is placed on a little sterile hygroscopic cotton wool in a Freudenreich or Hansen flask. When filter paper is used, a small piece is folded once, then wrapped up in four or five thicknesses of filter paper, and the whole sterilised. After this a few drops of the thick yeast liquid are cautiously poured on the inner sides of the folded paper and one of the coverings wrapped round it; when the latter has absorbed the moisture it is removed and replaced by a second covering, and finally placed in the remaining coverings. This process must of course be performed carefully so as to prevent infection taking place. A pure culture may be transmitted in this way in an ordinary envelope; the duration of life is limited, however, to a few months. A pure culture may be preserved safely by the above-described preparation on cotton wool, and the cells also live longer than in the filter paper covers. Also most kinds of moulds may be kept alive for several years by these dry preservation methods.

Preservation of Bacteria.—For preserving pure cultures of bacteria there is no method which corresponds to the Hansen saccharose method. In bacteriological laboratories pure cultures are preserved on or in the particular nutrient medium in such a way that they are always renewed after a certain time, a very troublesome process. Bacteria in the spore condition can, however, be preserved in the dry condition in many cases. According to Hansen's investigations acetic acid bacteria remain alive in beer for several years.

Preservation of Ordinary Brewery Yeast.—In connection with the preservation of pure cultures, a few words may also be said about the preservation of ordinary brewery yeast. Experiments were made and methods described more than 100 years ago concerning these more or less impure mixtures. Thus, the yeast was mixed with ashes and the moisture removed from the mixture by means of a cloth, or the yeast was mixed with sugar or with pulverised wood or animal charcoal and the mixture then dried. Beer in a cold cellar was also employed as a preserving medium.

O. Reinke described a method some years ago. The well-washed and quickly pressed yeast is very rapidly enclosed in two sheets of sterile filter paper. The yeast is then pressed flat, rolled up again in a sheet of ordinary white blotting paper, sprinkled with traces of sterilised boric acid, and then pressed between sterilised asbestos plates to remove the water. The latter are subjected in a hermetically closing metal box to a strongly cooled air current, sterilised and dried by concentrated sulphuric acid. After thorough drying the packets are arranged in a metal receptacle in such a way that each is surrounded with a layer of cold sterilised burnt gypsum. Finally the metal boxes are soldered up.

Will has also made experiments of this kind. He washed and pressed the yeast and mixed it with one of the following substances: kieselguhr, asbestos, gypsum, scraps of filter paper, wood charcoal and wood shavings: the two latter gave the best result, especially the wood shavings. The drying was done as quickly as possible on an oven at a temperature between 25° and 48°, being begun at the lower temperature and continued at the higher. When the yeast was dry it was filled into tin boxes which were hermetically soldered and stored at a temperature of

2° to 7° C. Some of the specimens preserved in this way contained living cells after the course of eleven years, this happening both with culture yeasts and wild yeasts. Heron has also described a method quite recently.

In all these methods for preserving common brewery yeast the latter is washed and pressed. Different experimenters perform the drying in somewhat different ways. Whichever it may be, the entrance of bacteria and other foreign organisms during these manipulations cannot be avoided. Even when one begins with a pure yeast from the pure culture apparatus, a yeast is obtained in the end which is always more or less contaminated.

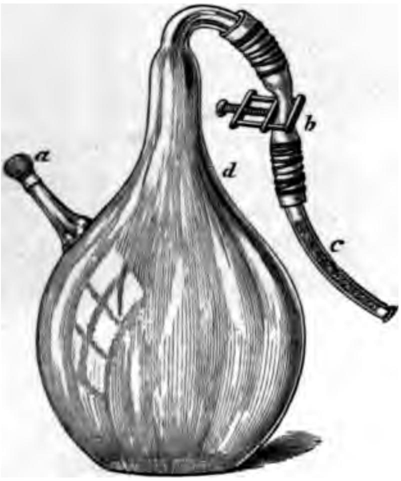

FIG. 46.—Hansen's Flask for the transmission of pure yeast cultures.

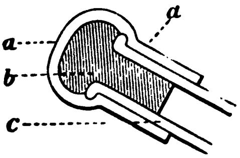

FIG. 47.—Section through the side tube of the Hansen Transmission Flask showing the method of closing.

Transmission of Yeast.—In sending small samples of yeast the methods described on page 117 using cotton wool and filter paper are taken advantage of. If a larger quantity of pure yeast culture is to be sent, which will be used in the liquid condition in the pure culture apparatus, Hansen recommends the employment of a flask the appearance of which may be seen from Fig. 46. The flask is made of strong thick glass and has a flat bottom. The yeast is passed into the flask through the side tube, after which the latter is closed. Fig. 47 gives a section of such a side tube when closed; *c* is the tube, the mouth of which is provided with a small collar, *b* a tightly closing rubber stopper, *a* a strong rubber cap tightly stretched over the stopper, being fastened at *d* by copper wire. The binding may be seen at *a* (Fig. 46). The rubber stopper must fit exactly and be easy to take out after the cap is removed. To add to the security the bent tube is divided into two parts, which are connected by a rubber tube; the latter is fastened to the glass tubes by wire; *b* is a pinchcock which can completely close up the tube, *c* is filled with cotton wool and used as an air filter when the yeast is poured off through the side tube. Both the rubber and glass tubing should be sterilised separately.

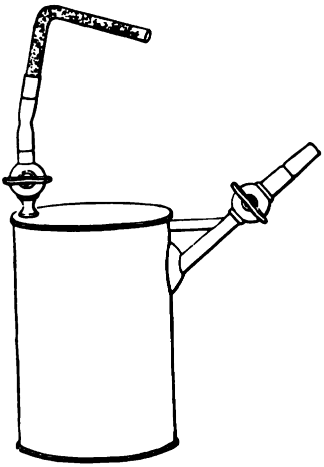

FIG. 48.—Jörgensen's Metal Flask for the transmission of pure yeast cultures.

A glass flask naturally has advantages over a metal flask as the contents are visible, which is of importance both in working with it and also in the transmission of yeast through the Customs, as is now so frequently done; but it is exposed to the risk of breakage during transmission if the packing is faulty. For this reason metal flasks have been frequently used of late as substitutes. Such for instance is the flask of Alfr. Jörgensen represented in Fig. 48. The principle is exactly the same as that of the Hansen flask.

The above-mentioned transmission flasks are constructed chiefly with regard to brewery requirements. Only small glasses or bottles are necessary for wine fermentation, as it is then generally a question of sending small quantities of yeast.

5.—*Preparation of Spore Cultures.*

Spore Cultures of Saccharomyces. — Even at the present day the statement sometimes occurs that a spore formation will be produced merely by sowing a little yeast on a moist gypsum block, a potato, or slice of carrot. In so far as the conditions of spore formation are treated, quite incorrect statements are even now not infrequently made, *e.g.*, that the yeast should be well washed beforehand, that the whole process depends upon a starving condition, etc. The old error of the formation of spores at a low temperature is now less frequently met with. Under the circumstances just mentioned it will, however, depend on chance, as regards most species of *Saccharomyces*, whether they form spores or not.

The essential part of the method does not consist in the use of any particular substratum, such as gypsum blocks, potato or carrot slices. The substratum on which the cultivation proceeds is in the main unimportant. A shallow layer of water in a culture flask, gelatine, etc., may be used with equal advantage, as will be shown later. The chief point lies simply in the use of a moist surface. But it is essential to the method that we should know the best conditions for favouring the function in question. These conditions were ascertained by Hansen and the essentials published in 1883, additions being made in later communications. (A more detailed explanation of the physiology of this function is given in the next section.) According to these investigations a copious formation of spores takes place, if (1) the growth consists of strong young cells; (2) a high temperature is employed (for most species about 25° C.); and (3) the supply of moist air is plentiful.

The technique of the Hansen method of spore formation is as follows: A flask containing wort is infected with a small quantity of the yeast species in question, shaken up and placed in a thermostat at 25° C.[1] In general a quantity of yeast sediment forms in twenty-four hours sufficient to perform the seeding out on the gypsum block. The supernatant fermenting wort is poured off, and a small quantity of yeast taken out by means of a pipette and spread in a thin layer on a dry, sterilised gypsum block in a glass dish (see Fig. 38, p. 65). It is important to make the yeast layer thin; with thick layers the oxygen of the air has no access to the lower cells. As soon as the layer of yeast is spread on the gypsum block, sterile water is poured into the dish until the gypsum block is immersed to about two-thirds of its height.

The addition of water is made by means of the water holder described on page 67 (Fig. 40). During this manipulation the cover of the glass dish must not be raised any higher than is necessary and the whole operation must be done as quickly as possible, because gypsum block cultures are very easily infected from outside. However, if the operation is skilfully done the infection is inconsiderable. As soon as the gypsum block is soaked with water, which is recognised by the glistening of the yeast layer, the culture is set away at the desired temperature.

The use of gypsum blocks was proposed by Engel. Similar substrata were recommended later by others, *e.g.*, earthenware cubes by Elion and "chamotte" blocks by Wichmann. According to experiments made by the author the latter are very inferior to the gypsum blocks, the formation of spores beginning later and the number of spore-bearing cells being fewer than when gypsum is employed. The porcelain cubes mentioned were found to be almost as good as the gypsum blocks.

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[1] The culture instead of standing for twenty-four hours at 25° C. may be left for forty-eight hours at the room temperature. If the growth is old it is advisable to freshen it once or twice at the ordinary temperature before it is used. In determining spore curves this has always to be done.

If it is desired to obtain a bacterium-free spore culture of a *Saccharomyces* according to Hansen, a thin layer of water may be worked with in a Freudenreich or Hansen flask or in a moist chamber, *e.g.*, Ranvier's chamber, with access of air ; or a seeding out on gelatine without addition of nutrient substances may be also used. Good results have been obtained from shallow water layers in flasks and moist chambers.

In many cases somewhat more copious formation of spores may be obtained on the gypsum blocks than in thin water layers ; if it is desired at the same time to protect the gypsum block culture from infection, it may be placed, according to Schiönning, in a Hansen flask (see Fig. 39, page 66). Sterile water is then added from another Hansen flask, the two side tubes of the flasks being connected. The yeast, on the other hand, is put on the gypsum block by means of a pipette through the neck of the flask.

It has been shown on page 99 that the influence of light may also be employed in the preparation of bacterium-free spore cultures of saccharomycetes.

The spores of saccharomycetes may sometimes be confused with other formations, especially with fat or oil drops, which are frequently found inside yeast cells. (If the preparation is treated with perosmic acid it may be easily ascertained if the bodies in question are of a fatty nature, as they then become brown or black. Fatty particles dissolve also in alcohol and ether, and are again precipitated on the addition of water.) The practised microscopist will, however, soon learn to distinguish spores from other objects. There is no decisive colour reaction for spores; they are usually stained by means of Ziehl's carbol fuchsine (see page 88) and retain their colour after the preparation has been decolorised with dilute acid. Experiments made by the author have shown further that spores are sometimes not stained by this process, and that on the other hand particles other than spores may be stained. Thus the method is uncertain. The mode of formation, the anatomical structure and germination furnish reliable characteristics for distinguishing whether a particle is a spore or not.

Spore Cultures of Bacteria.—There is no perfected method for inducing spore formation in bacteria similar to that which Hansen has described for saccharomycetes. In nearly all species of bacteria, spore formation occurs without using any special method of cultivation, being brought about merely by allowing the cultures to remain after the substratum has become poor in nutriment or has become unsuited for the growth from any other reason (as, *e.g.*, by the accumulation of fermentation products).

Spore Cultures of Moulds.—Zygospore formation is a phenomenon frequently observed in the *Mucorineæ* (see section III.). The conditions of this are still unknown, and there is therefore as yet no definite method of producing it. Bainier states that *Mucor racemosus* forms zygospores on gypsum blocks which are placed in dextrose solution. The author has tested this statement, but has obtained no positive result.

In the *Mucorineæ* and *Aspergilleæ* sporangia and conidia respectively are always formed when the mycelium grows on the surface of the culture medium, and when the latter is in other respects in a fit condition to act as a food. A mycelium immersed in liquid forms neither sporangia nor conidia.

In *Aspergillus*, ascospores are only known in those species which are classed under *Aspergillus glaucus* and *A. repens* (see Section III.). Although the particular conditions of ascospore culture are not known for these species, yet it is easy to produce them, as such spores always form when the culture is allowed to stand.

In *Penicillium glaucum*, which likewise comprises several species, a formation of sclerotia precedes the formation of ascospores (see Section III.). These sclerotia were obtained by Brefeld by infecting coarse bread, free from sourness, with conidia, and placing this between two glass plates which were pressed tightly together. Sclerotia developed in the course of three weeks. They were then washed and spread on moist filter paper, after which asci developed in their interior. In this species also the exact conditions are unknown.

6.—*Preparation of Film Cultures of Saccharomyces.*

Since film formation in the saccharomycetes plays a considerable *rôle* in the characterisation of the species, it will be necessary in many cases to prepare film cultures. The conditions for a vigorous film formation are, according to Hansen, the seeding out of a strong young growth on a favourable culture medium to which air has free access, and the placing of the culture in complete quiet at a moderate temperature. Film cultures are best formed by seeding out the *Saccharomyces* in an Erlenmeyer or Pasteur flask half filled with wort, this being set away in an undisturbed position at the room temperature. Hansen, Will and others have determined the cardinal points for some species. When the optimum temperature is known this is of course employed.

7.—*Counting of Yeast Cells and Seeding with a Definite Number of Cells.*

It is sometimes necessary to determine the multiplying power of a yeast species under certain conditions, or it may be intended to seed a certain quantity of yeast cells in a culture liquid. For this purpose it is necessary to count the cells, and in doing so the following procedure is adopted, the details of which have been gradually evolved in the Carlsberg laboratory.

For cell counting in a liquid it is required to obtain an exactly average sample. If this is not obtained, then, of course, the counting is of no value. The average sample is obtained by vigorously and repeatedly shaking up the flask with the culture, and taking from it, by means of a graduated pipette, a small measured sample, which is then put into a test tube. This operation is repeated. The withdrawal by means of the pipette must be done quickly, so that the cells do not begin to settle before the sample is removed. If the culture is in a flask provided with a side tube, the specimen can, of course, be poured out into a glass from which small samples can then be easily taken by a pipette. As soon as the sample is withdrawn, and it is desired to retain unchanged for some time the number of cells present at the moment, the culture must be set away on ice, or at a very low temperature; otherwise an increase in the number of cells may take place during the counting, which requires an appreciable amount of time. In order to obtain a reliable result, two samples are withdrawn, the one to check the other, and, for the same reason, several drops are examined from each specimen. Each sample is treated as described in what follows.

In many cases the cells to be counted are in wort. But since cells present in wort are hardly separated at all by mere shaking, and whereas this liquid is very inclined to form froth when shaken, and an increase of the cells in the sample withdrawn must be prevented during the counting, the samples are, according to Hansen, treated with dilute sulphuric acid (1 part concentrated sulphuric acid and 10 parts water). This furnishes, in addition, a liquid in which cells do not sink to the bottom too quickly, an important point when single drops are taken out for counting purposes. Supposing, for instance, that a test tube contains 3 c.c. of a sample of wort with yeast cells, it will be necessary in most cases to treat this with exactly 1 c.c. of dilute sulphuric acid. The dilution should not be proceeded with further than is absolutely necessary, since the observed number of cells must, of course, be multiplied by the dilution coefficient, and experimental errors are consequently increased. Besides, the presence of too few cells (or of too many) increases the difficulty of counting.

In counting, the counting chamber described on page 32 is employed (see Figs. 7 and 8). After the test tube with the average sample and the sulphuric acid has been subjected to a prolonged and vigorous shaking (this being done most easily by placing the thumb over the mouth of the test tube), a sample is taken out by means of a fine pipette as quickly as possible (before the yeast cells sink to the bottom) and a drop of the contents rapidly placed in the central part of the counting chamber. It cannot be too often repeated that it is absolutely essential to work with rapidity; otherwise it may happen that the cells in the pipette sink to the bottom and the drops then contain too many cells. The cover glass is put in place immediately the drop has been deposited in the counting chamber. The drop ought to be so large as to touch the cover glass, but not so large as to be pressed out by the cover glass over the edge into the surrounding space; if this happens the chamber should be carefully cleaned, dried and provided with a fresh drop. As soon as the cover glass has been put in position, the chamber is laid under the microscope, and if a hæmatimeter is being used as counting chamber, the "net" eye-piece is required. It is not advisable to use a greater magnification than is necessary. After waiting a short time, the counting is proceeded with when all the cells in the preparation have sunk to the bottom. The "net" eye-piece consists, as described previously, of a large square divided into sixteen or twenty-five smaller squares, the latter being used as aids in counting. The cells inside the large square are counted; it does not matter how the cells lying on the side lines of the square are counted, if the same rule is always followed. The same also applies to the counting of (apparently) dead cells and of buds which are still in connection with the mother cell. Many squares in each preparation may be counted by displacing the hæmatimeter. It is to be recommended, always to count a certain number of squares, *e.g.*, ten—two in the middle and eight along the edge of the drop. As soon as these ten countings are performed, the hæmatimeter is well cleaned and dried, the second test-tube well shaken and then a drop taken from it and counted in the same manner. This alternation is repeated until a constant average is obtained.

In the following example, which is from a counting made by the author, the exactness of the method is apparent. When it is not necessary to determine the number of cells in a given volume, the same unit of volume is always employed, *viz.*, that of a column of liquid of which the base is the large square of the "net" eye-piece for the particular magnification employed, the height being the thickness of the perforated cover glass.

The mixture, 3 c.c. of wort with yeast cells and 1 c.c. of sulphuric acid, gave the following results:—

Square.	Sample 1.		Sample 2.	
	1st Drop.	2nd Drop.	3rd Drop.	4th Drop.
1	23	10	28	13
2	22	20	20	24
3	19	28	19	21
4	10	19	22	14
5	14	24	32	18
6	27	26	25	20
7	20	14	21	19
8	18	25	13	34
9	12	20	17	23
10	27	14	20	16
Average	19·2	20·0	21·7	20·2
	Cells in each large square.			

As these four averages are nearly the same it is not necessary to count more drops. The mean of the four averages is $\frac{81 \cdot 1}{4} = 20 \cdot 275$ cells per unit of volume. But since the wort was diluted with sulphuric acid (4 parts of the mixture contain 3 parts of wort with cells), the actual number of cells in the volume in question is

$$\frac{20 \cdot 275 \times 4}{3} = 27 \text{ cells.}$$

If the counting has not been done with precision, or if the material does not allow of an exactly average sample being withdrawn, the result will not be so good.

If, as most frequently happens, the question is only one of determining the relative number of cells in several cultures, as in the foregoing example, or in the same culture after the lapse of varying periods of time (the multiplying power of the cells), then of course the same hæmatimeter or the same counting chamber, the same microscope, length of tube, magnification, and "net" eye-piece must be used each time in order to make a proper comparison.

When the number of cells is very large, dilution with the sulphuric acid must be carried further, often to four or five times the original amount of wort.

If the actual number of cells in a certain volume is to be calculated, the size of the space unit must be determined. It is then necessary to know the height of the column of liquid, *i.e.*, the thickness of the perforated cover glass. The hæmatimeter designed by Hayem and Nachet has one with a thickness of 0·2 mm., but that in the Zeiss hæmatimeter is usually 0·1 mm. The value of the square in the "net" eye-piece for the magnification used must further be known, or squared cover glasses are used of which the size of the squares is known. In Thoma's chamber the column of liquid is 0·1 mm. high and the large square etched on the bottom of the chamber contains 1 sq. mm. The volume of the liquid prism, of which the base is the large square, is thus 0·1 cubic mm.

When it is intended to sow a definite number of cells, water is usually added to the yeast to be used as sowing material, the cells being thus more easily separated from one another on shaking; also no appreciable increase of the cells takes place, especially if the flask is subjected to a low temperature after the sample has been withdrawn. The yeast is therefore shaken up vigorously and continuously with sterile water, and an average sample removed in the manner described above. There are three different cases to be now considered, *viz.*, (1) when we only wish to know how many cells are present in a certain portion of the water-yeast mixture; (2) when it is intended to inoculate a previously determined number of cells into the liquid to be dealt with; and (3) when it is desired to sow so many cells, that after the seeding the definite number of cells desired may be present in an arbitrary space unit, *e.g.*, when making comparisons of the multiplying powers of two species. In the first two cases it is required to determine the actual number of cells which are to be seeded, and no attention is paid to the quantity of liquid inoculated; in the last case it is only required to know the relative number of cells, but regard must be had to the quantity of liquid seeded. Finally the following must be remembered: If there is to be a definite volume in the flask after seeding, then, in the case where the seeding is not to be made in water or where the concentration of the liquid is of some account, no water must be used in shaking up the yeast. In this case the same culture liquid must be employed. The same quantity of culture liquid is then removed from the flask before seeding as will be added when seeding takes place.

The procedure in the above three cases is as follows:—

1. After shaking, a drop of the water is placed in the hæmatimeter or in the Thoma chamber, and the number of cells is determined in the usual manner. On seeding a measured portion of the water mixture we thus know how many cells have been sown.

2. As above. After the counting it is determined by calculation how much of the mixture must be taken out in order that a definite number of cells may be sown.

3. As above. In counting we learn, for example, that a cells are present in a certain volume. It is here necessary to know the quantity of culture liquid in the flask to be inoculated; assume this amount to be p c.c. If it is desired to seed so many cells that there will be a_1 cells per unit of volume, the number of cubic centimetres x of the water-yeast mixture, which must be added in order to arrive at this, is found from the following equation: $\frac{a}{a_1} = \frac{p+x}{x}$, or

the number of cells in the water mixture (the seeding liquid) has the same proportion to the number of cells after seeding as the whole amount of liquid after seeding has to the amount of the seeding liquid. The quantity of liquid in the flask after seeding has taken place is thus $p + x$.

From the given equation $x = \frac{a_1 p}{a - a_1}$. **Example**: It is found that the seeding liquid contains 75 cells per unit of volume and the flask to be infected contains 70 c.c. of wort, and it is further desired to have 5 cells per unit of volume after inoculation, accordingly $x = \frac{5 \times 70}{75 - 5} = 5$ c.c. to be withdrawn from the seeding liquid. The result may be checked by another counting after seeding. If the result is incorrect either more liquid or more cells must be added. But in exact work this contingency does not arise.

Suppose it is wished to sow a_1 cells of a yeast species A and b_1 cells of a species B in a flask containing p c.c. of culture liquid, from two seeding liquids containing a and b cells per unit of volume respectively, the number of cubic centimetres, x and y, to be sown from A and B respectively is found from the following two equations:—

$$\frac{a}{a_1} = \frac{p+x+y}{x} \text{ and } \frac{b}{b_1} = \frac{p+x+y}{y},$$

the quantity of liquid after infection being $p+x+y$; from this we find

$$x = \frac{a_1 bp}{ab - a_1 b - a_1 b_1} \text{ and } y = \frac{ab_1 p}{ab - a_1 b - a_1 b_1}.$$

Combinations of the above three cases may of course occur, but from the explanations given here it will not be difficult to solve them. It would lead us too far to go into more detail.

8.—*The Biological Analysis of Yeast.*

Preliminary Investigation.—The biological analysis of a yeast specimen is guided, to a large extent, by its origin and the eventual use to which the yeast is to be put. In general it is no small labour to determine all the elements of a specimen of yeast. This is begun by several preliminary experiments in order to obtain a basis for the real analysis. With this in view a microscopical examination of an average specimen of the yeast is made. The shape and size of the cells are observed; further, whether many dead cells are present, as that affects the use of the yeast in practice. It can also be found whether there are any mould spores and bacteria present. The detection of the latter is simplified by adding dilute soda solution to the preparation, by means of which dead particles of organic-chemical origin are in general dissolved; this is the case especially with resinous and albuminous bodies. It is often difficult to determine by microscopical examination whether the bacteria found are living or dead, especially if they belong to the non-motile species.

Some information as to the constituents of the yeast specimen can also be obtained by putting a little of it in wort at 25° C.; the phenomena of fermentation are then observed on one hand (top or bottom fermentation), and on the other the time taken to form a film on the surface of the liquid. Lastly, a small sample is placed directly on a gypsum block at 25° C., and information is obtained in the course of a few days as to the conditions of spore formation.

Separation of the Various Forms in the Sample.—The actual separation of the different species in a particular sample is effected by means of a plate culture of a small average sample in wort gelatine. Yeast and mould fungi, but only a few bacteria, are thus brought to development. If it is then desired to recognise the bacteria also present, a gelatine or agar-agar preparation of either yeast water or peptone meat extract should be used in making the plate cultures. Plate cultures on wort gelatine are set away at 25° C. When the colonies have grown to a sufficient size they are examined microscopically and macroscopically. Those which exhibit differences are inoculated in wort and studied more closely. The yeast sediment formed in the process is examined under the microscope; cultures for the eventual film formations are set apart; experiments are made in which it is observed whether the fermentation is bottom or top fermentation; the amount of alcohol formed is determined, etc. A spore analysis is also carried out (see below).

The comprehension of these analyses, of course, rests substantially on the insight which has been acquired into the biology of the organisms concerned, and on the use of the scientific results brought out by research. The analysis has been perfected chiefly for brewery purposes.

In nearly all cases of analysis of a brewery yeast it will be a question of determining to what extent (1) wild yeasts, (2) bacteria, and (3) various species of culture yeasts, are present in the sample in hand. The problem will seldom be that of determining the species.

Hansen's Spore Method for the Analysis of Brewery Bottom Yeast for Wild Yeast.—In testing for wild yeast, Hansen's spore method is generally employed. An average sample of the yeast is cultivated in wort at 25° C. for about twenty-four hours: gypsum block cultures of the yeast sediment produced in this manner are prepared in the usual way at 25° and 15° C.; after forty and seventy-two hours respectively the blocks are examined; if spores are found, wild yeast is present. Holm and Poulsen have shown that by this method $\frac{1}{200}$ part of wild yeast can be discovered in a mixture with culture yeast. Later, G. Syrée proved, with the aid of the same method, the presence of the wild yeast species *Saccharomyces Pastorianus* III. in a mixture with culture yeast in which it amounted to only $\frac{1}{400}$ part of the latter; the two species had been cultivated for four days at 25° C. In another case the original mixture was $\frac{1}{900}$ *Sacch. Pastorianus* III. and $\frac{899}{900}$ Frohberg yeast; after they had been cultivated together for eight days, the presence of *Sacch. Pastorianus* III. was demonstrated in this case also by Hansen's spore method.

The wild yeast can be most easily obtained by infecting a wort flask with the yeast sample and placing this away at 25° C. or at room temperature. At the close of the first fermentation a specimen of the surface beer is taken out; Hansen's investigations have shown that at this period the greatest quantity of wild yeast is found in the position mentioned. A new wort flask is then infected with the surface beer, and gypsum block cultures are prepared from the new culture in the manner already described.

Application of the Spore Method to Top Yeasts.—While Hansen produced his method specially for investigating brewery bottom yeast, and the experiments described above on the sensitiveness of the method were performed with such yeasts, Alfred Jörgensen has shown that this method also gives good results with brewery top yeast. He showed further that it is necessary to perform the analysis at 12° C. on account of certain top yeast species. The method has also been used later in the other branches of the alcoholic fermentation industries.

The Tartaric Acid Method.—Often, however, the admixture of wild yeast is so small that the above method fails to find it. The tartaric acid method, also described by Hansen, is then applied. An average sample of the yeast is placed in a 10 per cent. aqueous solution of cane sugar to which 4 per cent. of tartaric acid has been added, and this culture is set away at the room temperature. This cultivation is repeated four times after every twenty-four hours, or the culture is put away at 25° C. and recultivated twice at twenty-four hour intervals. A wort flask is inoculated from the last culture; then ordinary gypsum block cultures are prepared at 25° and 15° C. with the yeast thus produced, and these cultures investigated. In this manner quite small traces of wild yeast can be detected. In all cases a microscopical investigation is, of course, also carried out.

In the examination of cultures on gypsum blocks regard is paid to the appearance of the spores, the spores of culture yeasts generally containing a less refractive plasma with vacuoles, thus having an empty appearance, whilst the wild yeasts exhibit a strongly refractive plasma.

The above mentioned analysis of yeast by means of spore cultures on gypsum blocks can be simplified if the culture yeast in hand forms spores with extreme difficulty or not at all, as then the simple detection of the spores on the gypsum blocks at 25° C. is sufficient confirmation that the sample contains wild yeast or a foreign culture yeast.

Sporeless forms of the saccharomycetes can be prepared by the Hansen method, described in Section III.; the analysis is therefore simplified if a culture yeast of this kind is employed in practice. Since the asporogenous varieties of the saccharomycetes also form no films, this provides an additional means of detecting the presence of sporogenous, that is to say, foreign species; a wort culture being set aside to determine if film formation takes place.

Analysis for Bacteria.—In testing yeast for living bacteria an average sample is placed in yeast water and preserved at 25° to 30° C. When acetic acid bacteria are being sought, the beer can be kept at 32° to 33° C. if the yeast sample to be examined is still in beer, or if that is not the case, a little of the yeast can be placed in beer and then subjected to this temperature. Should living acetic acid bacteria be present these are quickly developed by this process. If it is required to determine in a yeast sample the bacteria which are capable of developing in wort, then of course wort is used instead of the liquids named above.

Testing the Contents of the Pure Culture Apparatus.—The above methods for the biological analysis of yeasts are applied in breweries to test the contents both of the pure culture apparatus and of the fermenting vessels.

In the first case a sample of the top beer is taken from the pure culture apparatus at the close of the primary fermentation by the side tube *j* (see Figs. 50 and 51). Wild yeasts are then detected by means of the tartaric acid method, and bacteria in the manner already described.

Testing the Contents of Fermenting Vessels.—So far as the testing of the fermentation vessels is concerned, this may be confined chiefly to a microscopical examination of the fermenting wort, especially when the yeast produced is not to be used as pitching yeast. The appearance of the yeast species used will in general be readily recognised by daily practice, so that a foreign admixture will be detectable by mere microscopical examination. A special knowledge gained in this way is therefore of great advantage; but on the other hand it cannot be too strongly emphasised that a microscopical examination alone is not a perfectly reliable criterion, and this is especially the case when the culture yeast used is similar in appearance to a wild yeast (*e.g.*, has more or less elongated cells). Foreign culture yeasts which may have found entrance in certain cases will, as a rule, not be recognisable; it is then even more necessary to apply physiological methods.

The sample is taken from the surface of the beer in a sterile glass four to five days before the end of the primary fermentation. In the microscopical examination, the form of the cells is observed, and whether living bacteria, especially rod bacteria and *Sarcina*, are present. If it is wished to test for wild yeast, the sample is set aside till a yeast deposit has formed; the latter is placed on gypsum blocks and analysed according to the spore methods already described. The yeast is placed directly on the gypsum block because the wild yeast presumably present has just formed strong young cells at this juncture. At the same time a wort flask is inoculated with a little of the yeast, and the yeast generated here is employed next day in a new spore test. If wild yeast is found by this test, the yeast cannot be used for pitching. This holds also when *Sarcina* or other bacteria are present to an appreciable extent. Bacteria are always observed in the wort, but are usually dead. Wild yeast is likewise always found in practice in small amounts; when it cannot be detected by means of the ordinary spore method there is no reason for apprehension.

The table on page 139 is given as an example of the journal of a fermenting cellar.

Lindner's Drop Culture. — P. Lindner, in examining for wild yeast, uses the "drop" culture. This consists in taking out a certain amount of beer by means of a pipette and distributing the contents of the pipette, drop by drop, (generally 50 drops) on the bottom and the cover of a Petri dish. It is thus known what quantity there is in these 100 drops. The Petri dish is placed in a thermostat at 25° C., or left at the temperature of the room. A development is visible in the drops after the lapse of several days. If the number of germs is too large, the liquid is diluted with wort to a suitable extent, before the dropping is performed. If it is wished to determine the principal kinds that are present, the upper dish is used, in the drops

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					Date.	Clarification.	Yeast Growth.	Bacteria.	Remarks.	Date.	Appearance.	Bacteria.	Remarks.
1898 10/6	23	983	Lager	14A	16/6	Good	Normal appearance	Some rods	No wild yeast in the spore test	21/6	Normal	Some rods	Used as stock yeast for vessel No. 31
10/6	25	984	Lager	14A	16/6	Not so good	Rather many elongated cells	Some rods	Wild yeast in the spore test		Condemned		
11/6	27	985	Lager	14A	18/6	Good	Normal appearance	Rods and sarcina			Condemned		

sterile glass four to five days before the end of the primary fermentation. In the microscopical examination, the form of the cells is observed, and whether living bacteria, especially rod bacteria and *Sarcina*, are present. If it is wished to test for wild yeast, the sample is set aside till a yeast deposit has formed; the latter is placed on gypsum blocks and analysed according to the spore methods already described. The yeast is placed directly on the gypsum block because the wild yeast presumably present has just formed strong young cells at this juncture. At the same time a wort flask is inoculated with a little of the yeast, and the yeast generated here is employed next day in a new spore test. If wild yeast is found by this test, the yeast cannot be used for pitching. This holds also when *Sarcina* or other bacteria are present to an appreciable extent. Bacteria are always observed in the wort, but are usually dead. Wild yeast is likewise always found in practice in small amounts; when it cannot be detected by means of the ordinary spore method there is no reason for apprehension.

The table on page 139 is given as an example of the journal of a fermenting cellar.

Lindner's Drop Culture. — P. Lindner, in examining for wild yeast, uses the "drop" culture. This consists in taking out a certain amount of beer by means of a pipette and distributing the contents of the pipette, drop by drop, (generally 50 drops) on the bottom and the cover of a Petri dish. It is thus known what quantity there is in these 100 drops. The Petri dish is placed in a thermostat at 25° C., or left at the temperature of the room. A development is visible in the drops after the lapse of several days. If the number of germs is too large, the liquid is diluted with wort to a suitable extent, before the dropping is performed. If it is wished to determine the principal kinds that are present, the upper dish is used, in the drops

Date of Pitching.	Vessel No.	Brew No.	Kind of Beer.	Stock Yeast.	Beer Samples.					Yeast Samples.			
					Date.	Clarification.	Yeast Growth.	Bacteria.	Remarks.	Date.	Appearance.	Bacteria.	Remarks.
1898 10/6	23	983	Lager	14A	16/6	Good	Normal appearance	Some rods	No wild yeast in the spore test	21/6	Normal	Some rods	Used as stock yeast for vessel No. 31
10/6	25	984	Lager	14A	16/6	Not so good	Rather many elongated cells	Some rods	Wild yeast in the spore test		Condemned		
11/6	27	985	Lager	14A	18/6	Good	Normal appearance	Rods and sarcina			Condemned		

of which the colonies are crowded at the lowest point, or are intimately mixed. All the drops are now touched with the finger, which has been cleaned and flamed previously, in order to obtain an average sample, which is then examined microscopically. To distinguish normal bottom yeast from wild yeast, the property is taken advantage of that the former aggregates in flakes, whilst the greater part of the wild yeast cells distribute themselves like dust in the drop. But since, as Lindner mentions, a part of the wild yeast also forms flocks, and the growths of culture yeasts on the other hand can assume a dusty appearance, other distinguishing features must be sought.

From Lindner's description of his method for analysing yeast in the brewery, it is seen that he draws his conclusions from the microscopical appearance of droplet cultures in hollow glass slips; this may be of use so long as it is remembered that cells of one and the same species, even when they are cultivated side by side in drops of liquid, often yield growths, the cells of which are so different as to seem to belong to several species. Under these circumstances culture yeasts may also develop cells with an appearance similar to one of the wild yeasts and *vice versâ*. Each characteristic is, as is known, subject to variation, but this is especially so with regard to the form of the cells. This analysis, when combined with the ordinary microscopical one, is of advantage to the practised specialist, whose eye for the form and general appearance of the yeast cells has been specially trained.

9.—*Hansen's Test of the Stability of Beer in Cask.*

Samples from lager casks are taken by boring holes in the cask. The place is first cleaned with spirit, and a try-cock [illegible]arly cleaned is placed in the hole. Portions of the [illegible] are drawn off from the upper, middle and lower layers, [illegible]ples being taken from these three different parts of the [illegible]d in order to get, as nearly as possible, an average sample. [illegible] weak point in this and similar analyses consists in the [illegible]ing of the samples, which must give a correct average if [illegible] analysis is to be of any value. The beer analysis [illegible]scribed in the following is given chiefly for the purpose [illegible] testing the stability, a point of great importance to the [illegible]rewer; what concerns him is to have some idea how the beer will behave after the lapse of a certain time. Hansen has published the following description of the test: The beer is drawn off in sterile white glass bottles, which are then closed with sterile corks and placed away in a dark cupboard at the temperature of the room. As soon as the samples are taken, their smell, taste, clarification and colour are noted. It is also noted how long it takes to form an appreciable deposit, and further, how the latter behaves: whether it distributes itself easily through the liquid on shaking, so that this becomes turbid and opaque, or whether it forms flocks which quickly sink to the bottom without substantially affecting the transparency. Changes of this kind are caused by the presence of micro-organisms. If the liquid becomes gradually turbid and decolorised without having been shaken, disease bacteria are present. However, this takes place but seldom after the pure culture system is introduced. On the other hand a yeast sediment always forms after a certain time even in the best beer, and may arise from culture yeast or from wild yeast, but is most frequently due to a mixture of the two. Hansen has shown that wild species of yeast can produce diseases at this stage. For the rest, when speaking of stability, reference is made only to the formation of yeast sediment and not to bacterial diseases. A yeast species which gives a stable beer is, in this sense, one which not only increases to a small extent in the finished beer, but is able at the same time to suppress its rivals during fermentation. The latter obtains either because it is in a better condition than its competitors to suit itself to the conditions of nutrition and especially to take up the oxygen, or because it gives off, during its multiplication, products which act as poisons.

Hansen's work has shown that samples from the upper layers of lager casks produce a yeast sediment more quickly than those taken from other parts. It is to be remarked here that beer under brewery conditions is strongly aërated when drawn off (when not drawn off under pressure of carbonic acid); but this does not happen when these test samples are taken, and this materially affects the increase of the yeast. Hansen's work showed further that it is necessary to keep the samples at the temperature of the room and not at 25° to 27° C., since the yeast sediment is produced sooner in the former case than in the latter. Moreover, the varying conditions in practice will be naturally of great importance in such tests, and therefore do not admit of the establishment of a general rule. It is therefore necessary as regards separate breweries to fix upon a standard which is obtained by experiment and analysis, and which is not changed so long as the same yeast is used, and so long as there are no great changes introduced during the manufacture of the beer.

In practice the following procedure is adopted when the production of a well-stored lager beer is in view: A sample is drawn about two months after the beer is casked. In doing this, white sterile bottles with sterile corks are employed. They are set away in darkness at the room temperature and observed several times in the course of a fortnight. It is noted how soon a sediment forms; if the sediment is considerable, its constituents are investigated. If the [illegible]lity does not appear to be satisfactory, a sample may [illegible] taken before the beer is drawn off, and this is treated [illegible] the same manner. When the drawing off begins, samples [illegible] again taken from the same casks, but in ordinary bottles, [illegible] sterilised ones. They are treated as by the retailers, [illegible]ing, for example, shaken up. Otherwise the examination is the same as that given above.

The table given on page 144 will serve as a specimen of the data entered in a lager cellar journal.

10.—*The Biological Analysis of Water, Air and Soil.*

In biologically examining water, air and soil, it is of fundamental importance to deal with an average sample; otherwise the analysis has little value. This is, however, very difficult, and in addition to this the organisms in water, air and soil vary considerably, with respect to number and species, with the time of year; it is therefore necessary to perform a large number of analyses at different times in order to obtain a knowledge of the actual flora of the micro-organisms and their relative proportions. Hansen's investigations mentioned in the next section may be cited as an example of such a series of analyses, which had as the object of investigation the circulation in nature of saccharomycetes, and especially of *Sacch. apiculatus*, and, above all, to discover what organisms are present in the air at different times of the year.

Principles of the Technical Analysis of Water, Air and Soil.—The manner in which a biological analysis of water, air and soil should be carried out depends upon the object of the analysis. A chief principle is the separation of the germs in sterile water and seeding from the mixture. If the question is to find all the species of micro-organisms present in a sample, the undertaking will be a very difficult one, especially as regards bacteria. For

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Cellar No.	Cask No.	When Filled.	Kind of Beer.	Remarks.	Stability—Test I.					Stability—Test II.				
					Date.	Taste and Smell.	Clarification.	Stability.	Sediment consists of	Date.	Taste and Smell.	Clarification.	Stability.	Sediment consists of
14	1375	18/12/98	Lager		28/2/99	Good	Good	Eight days, clear, very slight sediment. Fourteen days, clear, fairly large sediment.	Normal yeast cells, only isolated sausage-shaped ones, very few rod bacteria.	1/4/99	Good	Good	Eight days, clear, slight sediment. Fourteen days, clear, fairly large sediment.	Same as before.

isolating the different micro-organisms, several culture media are employed so that all kinds may develop. We will not, however, treat of such analyses, as they do not usually occur in practice. The questions here arising usually tend only in one direction. In the analysis of water for brewery purposes, information is generally sought for as to the micro-organisms of the water which are detrimental to the working of the brewery; the non-injurious forms are of no importance in practice, and are therefore left out of consideration. The culture medium employed in the brewery, *i.e.*, wort and beer, is used for such investigations. This simple principle had to be insisted on at one time by Hansen, as it was neglected by many workers.

The Hygienic Analysis of Water (after Koch) is performed with meat-extract peptone gelatine as culture medium; a certain quantity of the water is distributed in the latter and the number of colonies developed from it is determined. In analogy with this an air analysis is sometimes carried out| as well by drawing the air over culture gelatine.

The object of these methods is to develop as many germs as possible. They are, therefore, also used to test the efficiency of a filter by subjecting a certain quantity of water to plate culture before and after filtering, and afterwards comparing the number of germs developed in the two cases.

Water Analysis for Brewery Purposes (after Hansen). — A technical biological analysis of water for industrial purposes is made by sowing a certain quantity of the water in sterile wort, must, etc. An example of such an analysis of water for brewery purposes as carried out by Hansen may be described here. The questions to be answered are the following: How does the water behave towards wort and beer? How rich is it in such micro-organisms as can develop in these liquids, and are there among them such species as can cause dangerous disturbances in practical operations?

From the analyses given below will be seen the differences which results may exhibit according as the one or the other of the above-mentioned methods is used. Hansen found by his analyses the following numbers: While cultures in beer always gave 0 growths and a simultaneous series of experiments with wort 0, 0, 6·6, 3 and 9 growths per 1 c.c. of water, he found when Koch's meat-extract peptone gelatine was used under the same conditions and with samples of the same water, 100, 222, 1,000, 750 and 1,500 growths per 1 c.c. of water. This shows that the Koch method is inapplicable to such brewery analyses.

The procedure is therefore as follows: When, *e.g.*, the tap water in a brewery is to be analysed one begins by carefully cleaning the taps and tubing of the water supply, using all precautionary measures. The tap is then opened and the water allowed to run for some time, *e.g.*, one hour, before samples are taken. This ensures the washing out of the piping. The difficulty here, as in all biological analyses, consists in getting an average sample. If the water sample is not for transmission, but is to be analysed on the spot, sterile Chamberland flasks can be used for this purpose; if, however, the water sample is to be despatched, sterile bottles with glass stoppers are employed, the sample being packed in ice.

After the sample of water has been well shaken up, a small quantity is carefully withdrawn by means of a sterile pipette. The water is inoculated into sterilised wort and sterilised beer. In order to observe with greater ease the development of organisms that may be present it is best to employ a perfectly clear wort without sediment. Freudenreich flasks are the most suitable ones to use, as the number to be used is large, and larger flasks would take up too much room.

It is obvious that the power of preventing the growth of many organisms which wort and beer possess in virtue of the constituents derived from hops, and acid, etc., fails if too great a quantity of the water to be analysed is added. Holm has determined exactly the quantity of water which can be added to lager beer wort (about 14 per cent. Balling) before this happens. According to this author 15 c.c. of wort can be treated with ⅛ c.c. of water, and 15 c.c. of lager beer with ½ c.c. of water, before the resisting power fails.

In many cases it would be an error to take so much water, since it may contain so many germs that the inoculation would give rise to too many growths. The water sample should on this account be diluted with a certain quantity of sterile water (or wort) or it may be added in smaller quantity to each flask. It is impossible to give a definite rule for this. In each case it is advisable to make a preliminary test in order to determine approximately the number of germs. A reliable result is usually obtained when one drop of the water is placed in each of 100 flasks with wort; these flasks are then set away at 25° C. The infecting of beer flasks can be dispensed with since Holm's investigations have shown that in his analyses organisms never appeared which were only capable of development in beer. Any growth which developed in the beer flasks was derived from such species as might easily grow in wort. In sowing a drop in each of 100 Freudenreich flasks containing wort, 5 c.c. of water in all are used, and in most cases this gives rise to not more than one growth in one flask.

If flasks inoculated in this way have stood for one week at 25° C. and during this time no development has taken place, then there was, in the water sowed, no germ capable of development under the conditions of working. If such growths are present in some of the flasks, the contents of the latter are investigated macroscopically and microscopically, the number of growths noted and the quantity per 1 c.c. of the water calculated.

It is of some moment in practical evaluation of the water to take note of the time after sowing at which development, especially of bacteria, takes place in the wort. For it is obvious that when they develop only after four or five days they must have been so feeble as to be capable of development with great difficulty or not at all under practical conditions. In the laboratory, conditions are far more favourable for development than in practice, since the rivalry with the yeast (and the low temperature) is wanting. The result of a water analysis will therefore always be such that rather more germs are found than would have reached development under practical conditions in spite of the attempt to copy these conditions as far as possible.

Wichmann lays special stress on the importance, in evaluating the water, of noting the time when the "destruction" of the liquid under test takes place. He proceeds by adding to each of four flasks charged with 10 c.c. of wort, 1 c.c., $\frac{3}{4}$ c.c., $\frac{1}{2}$ c.c., and $\frac{1}{4}$ c.c. respectively of the water to be analysed. These four flasks are numbered 1, 2, 3 and 4. The destructive power of a water is taken as equal to 100, if all four flasks exhibit development after the lapse of a day; this number is got by multiplying the numbers of the flasks by certain factors and adding the four products. If development takes place in the flasks after one day this factor is 10, after two days 8, after three days 6, after four days 4, and after five days 2. Thus if all four flasks are turbid after twenty-four hours, the destructive power is: $10 \times 1 + 10 \times 2 + 10 \times 3 + 10 \times 4 = 100$. If flask No. 1 shows development after two days, No. 2 after three, No. 3 after four, and No. 4 after five days, the destructive power is 1 × 8 + 2 × 6 + 3 × 4 + 4 × 2 = 40. From this it may be seen that Wichmann adds more water to the wort than Hansen and Holm in their analyses found to be advisable.

Schwackhöfer's Standard of Fitness of a Brewery Water.—Schwackhöfer has proposed the following scale as a standard of the fitness of a water for brewing purposes, twenty-five flasks being each infected with one drop of water. In those cases in which certain micro-organisms develop neither in wort nor in beer, he describes the water as specially good. If development takes place in 10 per cent. of the wort flasks at the most, but not in the beer flasks, the water is good; if development takes place in 50 per cent. of the wort flasks and in none of the beer flasks the water is fit for use; if micro-organisms are present in more than 50 per cent. of the wort flasks, and in at most 19 per cent. of the beer flasks, the water is only to be employed in cases of necessity, and finally if the percentage of flasks of both categories exhibiting growths is higher than that above mentioned, the water is unfit for brewery purposes. It must be remembered here that chemical analysis has been entirely left out of account.

Holm's Results.—In Holm's analyses of water from the Carlsberg Breweries, bacteria, moulds and yeast-like cells (*Torula, Mycoderma*) appear in the wort and beer, but no species of *Saccharomyces*. The presence of the latter is nevertheless not precluded, but their appearance in the water is at any rate rare. The species of moulds were especially numerous, not only in wort but also in beer; the same remark applies also as regards the number of growths. Bacteria were found along with these in the wort, whereas they appeared but seldom in beer. Yeast-like cells were rarely observed. Among the organisms observed by Holm in water the following may be named: *Rhizopus nigricans*, *Penicillium glaucum*, *Mycoderma cerevisiae*, *Bacterium aceti*, and *Bacterium Pasteurianum*. In addition, Jörgensen and Lindner have found *Sarcina*. The latter investigator found it frequently, and supposes that a prolonged existence of the *Sarcina* germs in water renders them more capable of germinating in hopped wort.

With regard to the importance of the micro-organism content of the water for brewery working, the germs present in the steeping water will be of little consequence in the manufacture of malt. There are, in fact, many germs on the surface of the barley corns, and the few which are added with the water are of little account. When the wort is boiled, these organisms are killed; they may, perhaps, be of some significance during mashing. The danger is naturally greatest in the fermentation and lager cellars: but other factors appear here, *viz.*, low temperature and the large quantity of healthy yeast with its suppressing power, which restrain the development of the water germs.

In carrying out a water analysis we must direct our attention to the cistern or the well from which the supply proceeds. Surface water, as is well known, contains a large number of germs, and these will increase to a large extent if the piping and the cistern are not kept properly clean.

Hansen's Analyses of Air.—It is more difficult to obtain an average sample of air than of water. The same principles hold here as in water analysis. Hansen in his time used flasks with wort for investigating the circulation of yeasts and the organisms of the air in the various parts of a brewery. Large numbers of these flasks were left open at different times of the year at those places where he wished to analyse the air. The so-called vacuum flasks were also employed for the same purpose. The latter are flasks charged with culture liquid which are sealed during the boiling of the liquid; after cooling there is an air-exhausted space over the culture liquid. If the sealed end is now broken off at the place where it is wished to examine the air, a certain quantity of air is sucked into the flask. The germs contained in this air are brought by shaking into contact with the culture liquid in which they then develop.

In his last treatise on this subject Hansen recommends the passing of a certain quantity of air through sterile water, which retains the germs and can be subsequently analysed. An air analysis is, in short, performed in the same manner as a water analysis. For this purpose graduated pipettes are used or, where possible, Miquel's flask, which is represented in the accompanying drawing (Fig. 49) in union with an aspirator. The latter, A, is a bottle with an outflow tube, *a*, near the bottom, to which rubber tubing with a pinchcock is fitted. The neck of the bottle is closed by a bung through which a glass tube passes which is bent

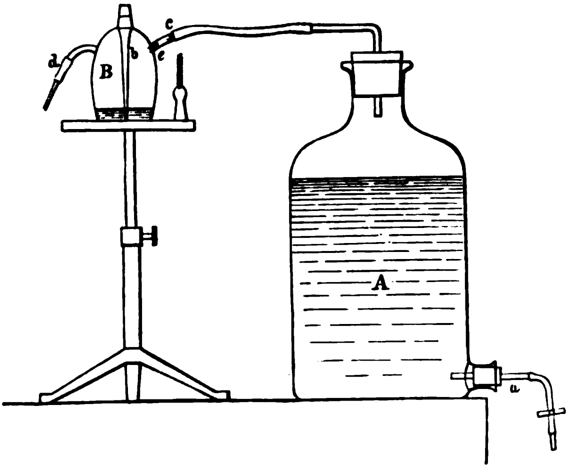


FIG. 49.—Miquel's Flask, B, in combination with an aspirator, A.

once and is connected with the Miquel flask, B. The latter is shaped somewhat like a Chamberland flask and is provided with two side tubes, *c* and *d*, but the neck is fitted with a glass tube, *b*, which reaches almost to the bottom.

During sterilisation, B carries a glass cap with a little tube filled with cotton wool (as in the Chamberland and Freudenreich flasks); the one side tube is likewise closed at *c* by a wool plug. At *e* a very loose wool plug is fitted which can be easily blown into the water in B at the end of the experiment. At *d* there is a rubber tube which is closed by means of a glass tube drawn out to a fine point and sealed. The flask further contains a measured quantity of water in such amount as to immerse the opening of the tube, *b*. In this condition the flask, previously sterilised in the dry state, is again sterilised. The whole apparatus is then set up at the place where it is desired to examine the air, the tube, *c*, being connected with A by a rubber tube.

The experiment now consists in sucking the air to be examined through the water in B, so that the germs are retained by the latter; this is done by opening the pinchcock, *a*, so that the water in A runs out. The air must not be drawn too quickly through the water; the water in A is therefore allowed to pass out at *a* drop by drop. As the experiment proceeds the pinchcock at *a* may be opened more and more as the flow of water gradually lessens. The wool plug, *e*, retains those germs which may be carried off by the air without being taken up by the water in B. It is obvious that the volume of air sucked through the water in B is the same as the volume of water which runs out at *a*. The quantity of water in A must therefore be known. This bottle, however, cannot be completely emptied through *a* while in an upright position, and this should be kept in mind when the quantity is determined. For this reason it is advisable to first pour as much water into A as will fill cover the inner opening of the tube *a*, and then to mark the level of the liquid in the flask; a volume of water equal to that of the air to be analysed is then added.

As soon as the desired quantity of air is passed through the water in B, the pinchcock, *a*, is closed and the cap placed on B; the tube, *c*, is then disconnected from the rubber. There are still some germs in the tube, *b*, which have not reached the water; the tube, *c*, is therefore blown through until the water rises in *b*. This is repeated several times until *b* has been washed out. Finally the wool plug, *e*, is blown or pushed into the water. B is now well shaken up, partly in order to distribute in the water the germs which are on *e*, and partly to effect a thorough and uniform distribution of the germs throughout the water. The procedure is then exactly as in water analysis, the water being dropped through *d* into the flasks containing culture liquid. This is done by cautiously breaking off the point of the thin glass tube which is fitted into the rubber tubing. The dropping can be regulated by holding the finger over the opening of the tube, *c*.

If there is no more water in B than is used for the inoculating, it is not necessary to know the amount of this water. If, however, only a part of this is used, which will probably be the most frequent occurrence, it is necessary to know how much of the total amount of water has been used. Suppose for example that 6 litres of air have been passed through the water in B, and that B was charged with 10 c.c. of water, and assuming that one drop = 0·05 c.c., it follows that if 100 flasks are inoculated with one drop each, 5 c.c. of water in all, or, in other words, the half of the water has been used, and therefore the number of germs in half the quantity of air, *i.e.*, in 3 litres, is determined. Sometimes it happens that the air is so rich in germs that it is necessary, as in water analysis, to dilute the water.

If it is required to quickly obtain some idea as to the purity of the air with regard to germs, Petri dishes with wort gelatine may be left open for fifteen minutes. The covers are then replaced and the plates put into a thermostat at 25° C. The germs then develop.

Hansen's Results.—In his researches on the micro-organisms of the air, Hansen had partly in view theoretical considerations, such as the circulation of species in nature, especially that of the saccharomycetes, and he partly followed out purely practical problems concerned with brewing. As regards the solution of the latter the air was examined in different parts of the brewery (fermenting cellar, cooling vessels, etc.). In connection with this, experiments were carried out to discover whether the vapours from the grains carried infection by means of the numerous bacteria they contain. Hansen arrived at the result that this does not take place; on the other hand, dried grains become dangerous in a high degree as soon as they are carried by the wind as dust in the air. Therefore it is not advisable to employ any apparatus for drying the grains in the neighbourhood of the brewery; for, according to Hansen, the bacteria are not killed by this drying; if, therefore, drying apparatus is set up in such a position that the dry particles with numerous bacteria can find their way on to the cooling vessels and into the fermenting cellars, much harm may be caused by this means.

Hansen further found that the purest air in the Old Carlsberg Brewery was in the fermenting cellars, this being due to the fact that the cellars are provided with cold air which has been previously purified. In analyses of the air in the fermenting cellars of other breweries which were without purified air bacteria were observed, among which were *Sarcina* and various species of wild yeasts, disease forms also being found. The cooling vessels are exposed fection from the air, especially at the time when sweet, y fruits are ripe, when the dust from the ground is y rich not only in yeasts but also in bacteria.

Analyses of air should be performed in breweries from e to time; a clear idea is thus obtained of the progress the different processes, and will in many cases avert shap. It is the air of fermenting cellars and coolers in rticular to which special attention must be paid.

Soil Analyses. — In soil analyses a small sample is aced in an Erlenmeyer flask containing a culture liquid osen with regard to the organisms to be sought. The ora of the soil is a very rich one, especially as regards bacteria and mould fungi. If saccharomycetes are to be ooked for, it is advisable to seed the soil sample in wort to which tartaric acid has been added, since this prevents to a great extent the development of most bacteria. In this case also it is desirable to allow the cultures to remain for a considerable time (about fourteen days) at 25° C., and then to prepare a second culture in wort, as saccharomycetes are generally tardy in their development.

It has also been proposed to mix a small quantity of the sample of soil with liquefied nutrient gelatine and then to prepare plate cultures. However, the result is in most cases bad, the number of germs in the soil being too large. Others prefer making a paste with the soil in sterile water; plate cultures are then prepared from an average sample.

These analyses, in common with all the foregoing, have the object of discovering the source of the infection which may take place in a brewery. The groundwork for this was furnished by Pasteur's and Hansen's investigations.

11.—*Hansen's Pure Culture System in Fermentation Industries.*

The Pure Culture System in Bottom Fermentation Breweries.—The following information on the introduction of the systematically selected yeast race into the bottom fermentation brewery is extracted from Hansen's *Practical Studies in Fermentation.* The starting point must be the yeast which has proved its superiority in working in practice, and has yielded that product which it is wished should be the future normal production of the brewery. Since the species which lends its character in great measure to the product will be present in superior numbers, it will also be the most easily isolated.

If there is wild yeast present in the stock yeast employed, it will, according to Hansen, be present only in small amount in the surface beer at the beginning of the primary fermentation, or it will be totally absent, while at the finish the reverse is the case. A sample of the surface beer is therefore taken just at the time when a frothy head has formed in the fermenting vessel; we are then certain that the race or species to be isolated is in preponderance, and this sample is used for the preparation of absolutely pure cultures, the starting point being made as usual from a single cell (see page 106). Preliminary fermentations are now made with these pure cultures in flasks in the laboratory. It is advisable to use the same wort as that employed in the brewery, and this should not be re-sterilised in the laboratory. The method of procuring this is described on page 76. While fermentation proceeds in the flasks, certain preliminary observations are made which will be of use later on. It is observed to what extent the wort remains clear, whether the yeast lies compactly on the bottom, and whether the beer has any peculiar smell and taste, etc. A microscopic examination and also a spore culture on a gypsum block are of course made; in fact, the characteristics of the yeast are investigated. It may happen that the growths in the flasks which contain pure cultures of the required species are nevertheless somewhat varied, although they belong to the same species. We are here confronted by individual differences, which may always be met with, and we must now make a choice from among these growths also.

After preserving some of the pure culture (on the preservation of yeasts, see page 114) partly in saccharose solution and partly in the dried condition on cotton wool, the procedure is as follows:—

Four or five 1 litre Pasteur flasks, containing ordinary aërated but sterilised wort from the brewery, are inoculated from the flask containing the perfectly pure culture; these are set away at the temperature of the room, and in a week they will contain a considerable yeast sediment; four such flasks will generally be sufficient, the fifth being really a reserve culture. After pouring off the beer, the yeast of each of these flasks is introduced into a Carlsberg vessel charged with about 7 litres of brewery wort. After one week as much yeast sediment will have formed in these vessels as is necessary to prepare stock yeast for 1 hectolitre of wort in the brewery. A vessel of 1½ hectolitre volume is then set up in the fermenting cellar; this is sterilised by means of a gas flame and charged with a hectolitre of aërated brewery wort. The contents of the four Carlsberg vessels are then poured into this vessel. If the partially fermented wort in the vessels is not to be added also, it is previously drawn off. In the latter case it is advisable to let the flasks stand a little longer, about ten days, so that the yeast may sink more completely to the bottom. If the brewery is some distance from the laboratory it will be always necessary to draw the beer off beforehand. The flasks mentioned above (see page 119) are then used for transporting the thin yeast liquid. The hectolitre of wort mentioned is fermented under the normal conditions of the brewery. If the result is satisfactory the yeast is introduced into practical working.

It is conceivable that a brewery may have operated continuously with a mixture of different species of culture yeast, and that the combination of the latter has formed the character of the product. It is on no account advisable to isolate these species separately and then to employ a mixture of them as stock yeast, for the relative proportions of the species cannot be controlled during fermentation. Besides, it would be far too much trouble in practice.

We have mentioned before that a pure culture yeast of this kind introduced into practice can only keep sufficiently pure in the fermenting vessels for a certain length of time. The resisting powers of the different races against wild yeasts and bacteria is extremely varied. It is, therefore, necessary from time to time to introduce fresh quantities of pure culture yeast into the brewery. There are, however, cases where a race has remained pure in the ordinary fermenting vessels of practice for more than a year without having been renewed.

The Hansen-Kühle Pure Culture Apparatus.—In order to have ready at hand the requisite amount of pure culture yeast, Hansen, in conjunction with Kühle, has constructed a pure culture apparatus for the continuous production in mass of absolutely pure yeast. A description of the apparatus is given below:—

The apparatus (Fig. 50) consists of three principal parts, *viz.*, an air pump, A, with an air reservoir, B, a fermentation cylinder, C, and a wort cylinder, D. The air pump receives the air through a filter and pumps it into the reservoir which is provided with a manometer and safety valve. The air can pass from the reservoir through the communicating pipes, which are provided with outlet taps for condensed water, through the cotton wool filters, *g* and *m* respectively, into the fermentation or wort cylinder.

The following parts are connected with the fermentation cylinder, C: (1) a doubly bent side tube, *c*, which opens under water in a vessel, *d*; (2) a glass tube, *f*, with marks (10, 20, 79), which indicate the amount of liquid in the cylinder; taps are fitted at *e* and *h*; (3) a stirring apparatus, *b*, to stir up and distribute any bottom yeast formed in the cylinder; (4) an outlet cock, *l*, for beer and yeast; (5) a short side tube, *j*, provided with rubber tubing and glass stopper: the pure

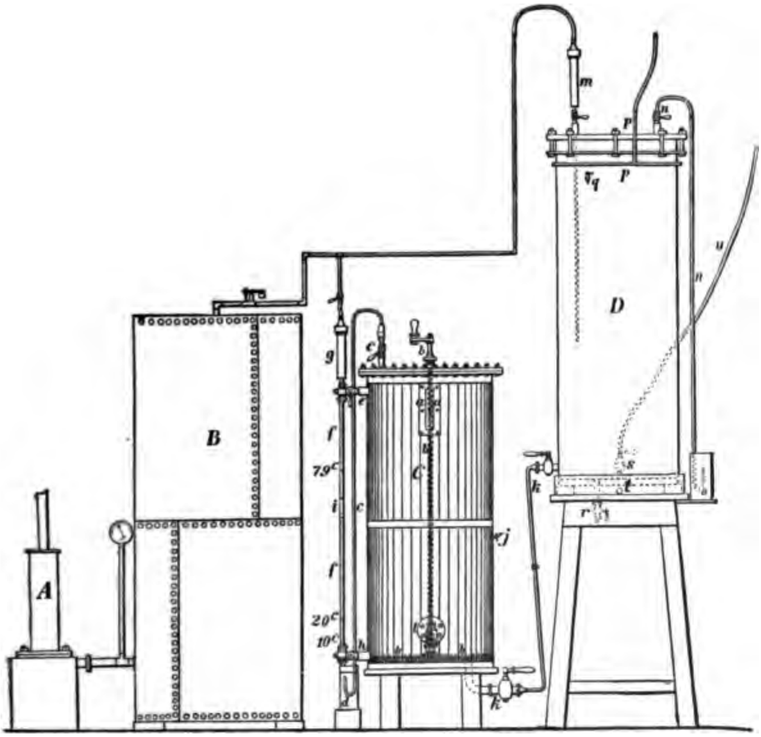


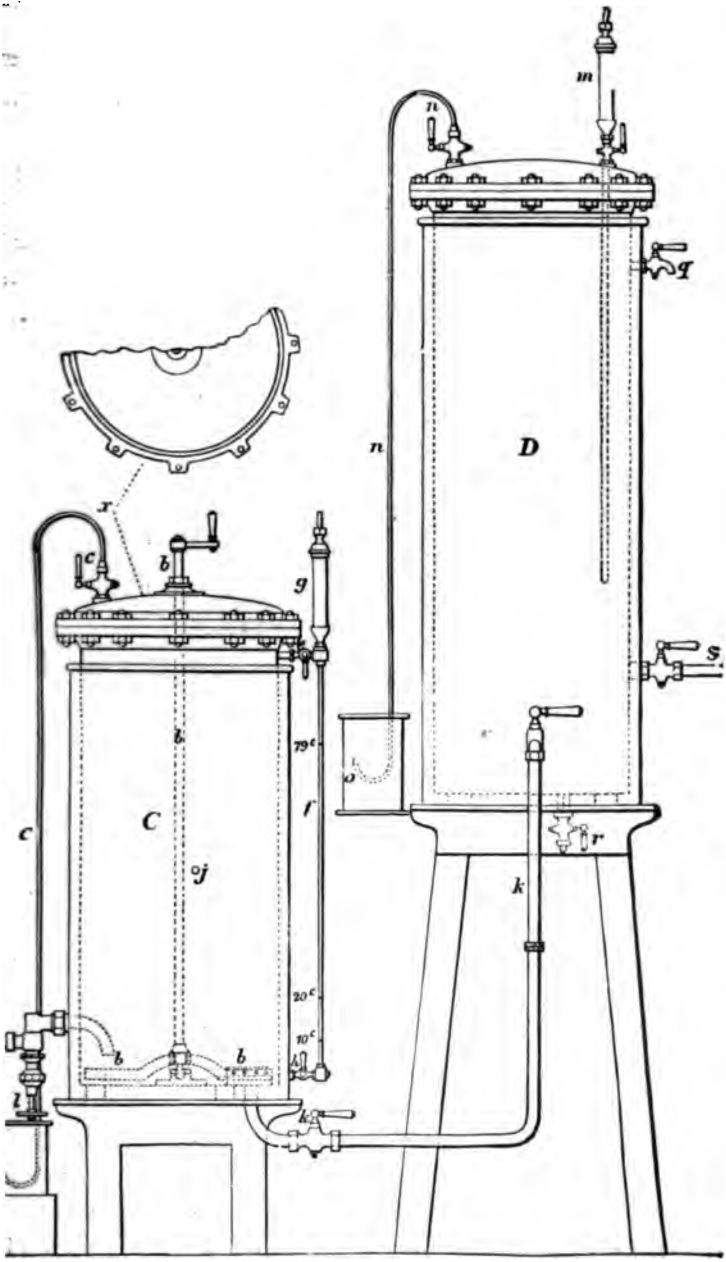
FIG. 50.—Hansen-Kühle Pure Yeast Culture Apparatus.

culture of yeast previously prepared in the laboratory is introduced through this tube, the side tube of the flask being connected with it in a flame in the ordinary way; (6) a pair of windows, *a*, set at an angle to one another, which are only added if requested, being best omitted as they are superfluous. Lastly there is sometimes a water jacket (Fig. 51) fitted round the cylinder when the apparatus has to be set up in a spot where the temperature must be regulated.

Connected with the wort cylinder, D, are: (1) a doubly bent side tube, *n*, opening under water in the vessel, *o*; (2) the stopcocks, *q*, *r* and *s*; *s* is in communication with the pipe through which the boiling hot wort is passed from the brewery into the cylinder; (3) a sprinkling ring and a cold water pipe, *p*, for cooling the wort. The sprinkling ring is perforated with small holes on the side next to the cylinder. The cylinder is fitted into a box which is provided with an outlet tube, *t*, and which receives the cold water flowing through the sprinkling ring. In Fig. 51 a water jacket is employed instead of this ring; it is more efficient but also more expensive. The wort and fermentation cylinders are connected by means of the pipe, *k*.

The outlet cock, *l*, is fitted so that infection from outside is obviated during tapping. The construction may be seen from Fig. 52 in which the cone valve is shown shut. The arrows give the direction of flow when the stopcock is screwed open. The construction of the lower part of the stirring apparatus, *b*, is given in Fig. 53.

When the apparatus is fitted up, it is first tested to see whether it is air-tight. For this purpose steam is led into the cylinder through the pipe, *k*, all other cocks being shut. As soon as it is found that the apparatus is steam-tight, it is sterilised by means of steam, all the cocks being opened in turn and closed after the steam has passed through for some time. When the apparatus is sterilised, the wort-



F. ROSENSTAND X. A.

FIG. 51.—Hansen-Kühle Pure Yeast Culture Apparatus.

cylinder is filled with boiling-hot sterilised wort from the brewery ; it is then cooled, aërated through *m* and, after this has taken place, a part of it is passed over into the fermentation cylinder by means of air pressure. The pure cultivated yeast from the laboratory is then added, the stirring apparatus set in motion, and later on more wort is added. As soon as the proper amount of yeast is formed, the beer is drawn off, and the sedimentary yeast stirred up and mixed with the small quantity of beer remaining ; this mixture is then taken out, to be used as pitching yeast for a small fermenting vessel (with about 8 hectolitres of wort). Wort is then added for the next culture.

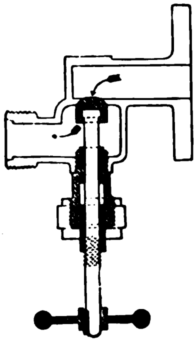


FIG. 52.—The Construction of the Outlet Tap of the Hansen-Kühle Pure Culture Apparatus.

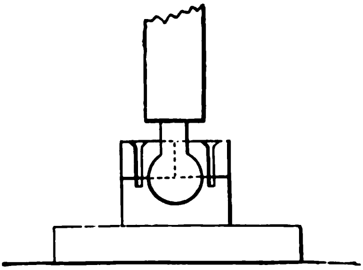


FIG. 53.—The Construction of the Lower Part of the Stirring Apparatus of the Hansen-Kühle Pure Culture Apparatus.

A detailed description and guide for the use of the pure yeast culture apparatus described here as well as of the pure culture system in general was given by E. Chr. Hansen himself in his *Practical Studies in Fermentation*, London, 1896. Exact directions are given which are the outcome of very comprehensive experiments, which he made in the Old and New Carlsberg Breweries, Copenhagen, as well as of extensive observations in these and other breweries at home and abroad. Some modifications have been made in the apparatus just described, which is the most extensively used form, and changes have been introduced by the following, among others: Bendixen, Bergh & Jörgensen, Brown & Morris, Elion, Lindner, Marx, Thausing and Wichmann.

However carefully one may work there is always the possibility of infection taking place in the apparatus by some unfortunate means. It is therefore necessary to subject the yeast produced in the apparatus to a controlling analysis from time to time (see page 137). The yeast in the apparatus should not be changed without good reason. If the apparatus is manipulated carefully it will remain free from infection for years.

As already stated, Hansen began his laboratory researches on pure culture yeast races in 1881, and carried out conclusive experiments in practice two years later in the Old Carlsberg Brewery. When the new reform had gained a firm foothold there and in several other bottom fermentation breweries, it naturally resulted in its extension to the other branches of the fermentation industry.

The Pure Culture System in Top Fermentation Breweries.—The first to experiment in practice with purely cultivated top yeast was Alfred Jörgensen, who, in 1885, introduced a yeast of this kind into a Danish top fermentation brewery with most satisfactory results. The method was the same as that described above, used by Hansen in bottom fermentation breweries, but in some cases it was found advisable to aërate the wort a little, as clarification proceeded somewhat slowly. Subsequently a large number of good species of beer top yeast were isolated by Jörgensen, Schönfeld and others, and the pure culture system introduced into numerous top fermentation breweries on the continent, although not to such an extent as in bottom fermentation breweries.

Modifications of the Hansen-Kühle pure culture apparatus have been designed by Jensen, Jörgensen, Kokosinski and Wilson for use in top fermentation breweries.

The Pure Culture System in Wine Manufacture.—The Hansen pure culture system has attained great importance in the preparation of wine. The systematic selection from the numerous wine yeast races is here probably of even greater significance than in the other branches of the fermentation industry. In this case the most varied demands are made on the yeast, which are probably directed for the most part to obtaining a better product from inferior material. In this domain also pure yeast has realised completely all reasonable expectations, although it cannot be denied that many expectations went beyond reasonable limits.

The first to use Hansen's system in wine manufacture was one of his pupils, L. Marx (1888); later on, Hotter, Mach, Müller-Thurgau, Portele, Seifert and especially J. Wortmann have extended the application of the system in this sphere.

The technique of pure culture in wine manufacture is somewhat different from that in the other branches of the fermentation industry. In a brewery, a sterilised liquid is dealt with, a wort; but it is not possible to sterilise must, as it would take up the so-called boiled flavour and the wine would thereby depreciate in quality. This circumstance entirely prevents the use of sterilised must in the preparation of wine. The germs present in must are, however, as a rule so weakened that they only develop after some time; a large quantity of a vigorous young growth of the selected pure yeast is therefore added at once to the must, and foreign germs are by this means suppressed. This action is assisted very considerably by the chemical composition of the must; and it is this also which is responsible for the fact that spontaneous fermentation has been advantageously employed for so long a time, a good product being obtained without sterilisation or centrifugalising.

According to Wortmann's researches the end products of fermentation, as well as the quantity of these, are the same, whether little or much yeast be added; but the more yeast added to the must, the quicker is the fermentation. It is best when fermentation proceeds quickly, as, in consequence, the foreign germs present in the must are more completely suppressed; the fermentation may, however, be too violent if an excessive quantity of yeast is added; the result of this is, that the must not only froths over easily and a part of it is thus lost, but, by the vigorous production of carbonic acid, bouquet substances are also carried off at the same time and the wine loses in quality.

With regard to the quantity of yeast to be employed, the following numbers given by Wortmann may serve as a guide: With light musts, *i.e.*, musts containing about 18 to 20 per cent. of sugar, one can rely with certainty on the fermentation being controlled by the pure culture yeast and consequently on a good result if from ¼ to ½ per cent. of yeast is added. By this is understood that to every 100 litres of fresh must is added ¼ to ½ litre of a must brought into vigorous fermentation by means of a pure yeast. Good results are also obtained with the larger addition of ½ to 1 per cent. But Wortmann found the effect of still larger additions of yeast to be too great with light musts. With heavy musts the addition of yeasts may be much larger, *i.e.*, up to 1 per cent. or even more, without fear of bad results. If it is desired to re-ferment wines not thoroughly fermented, or sugared wines, the addition of pure yeast should be still greater, in fact 2 per cent. or over. The same holds good for the employment of pure yeast in the preparation of sparkling wine. If wines which have stopped fermenting are to be forced with pure yeast, Wortmann recommends a yet greater addition of yeast, up to 10 per cent., according to circumstances.

The temperature of the fermenting room requires careful watching; it should be kept lower than it is otherwise usual to keep it, so that in fermentations to be carried out on the large scale with pure yeast, the fermenting may not be too violent.

It is, in addition, important to employ the yeast at the proper stage of development. The yeast must be in such a condition that, when placed in the must, it can immediately continue its development, so as to obtain the mastery in as short a time as possible. Therefore the practitioner obtains from the laboratory small quantities of yeast, which he then increases in definite quantities of must.

Since the number of wine yeast races brought into use is large, and the fermentation of grape must is limited to a few weeks in each year, a pure culture apparatus such as that which has been described for use in brewing for the continual production of yeast in mass is not applicable in this branch of work. Laboratories would require to have a large number of such pure culture apparatus, and even if they possessed them it would be impossible in the short time at disposal to produce the quantity of yeast necessary for the work. Consequently stations and laboratories dispose of the pure culture yeast in small quantities which the practitioner then increases for himself.

In cultivating the yeast, laboratories employ sometimes the concentrated must mentioned on page 80 and sometimes a must pressed from home-grown grapes; the former is diluted with water. In cases where a specially vigorous yeast is required, *e.g.*, in re-fermentations and fermentations of unfinished wines, the employment of well-nourished, non-aërated yeast is to be recommended.

Pure yeast is supplied to wine producers in a thin d condition by the stations, *e.g.*, by that in Geisenheim by the fermentation laboratory at Klosterneuburg; the contain as much yeast as is produced in 0·5 to 2 litres culture liquid.

The Geisenheim station has given the following directions for the use of this pure culture yeast: The flask with pure culture ought not to be more than two weeks old the very most. Some days before the beginning of the final vintage, about 12 litres of freshly prepared, not fermenting must from good, ripe, sound grapes are allowed to boil for about five minutes, being skimmed carefully meanwhile, and then allowed to cool down to the temperature of the room, the pot being covered. After cooling, the contents of the yeast flask should be poured into the must, the flask washed out several times with must, and the pot securely covered again, and placed away in a dust-free situation at the temperature of the room, until the must, after two to three days, exhibits violent fermentation. The must thus brought to fermentation is then put into the fresh must to be fermented, the quantity of which depends on the conditions at the time.

The selection of the pure yeast is of the highest importance in the preparation of sparkling wines. It affords the certainty that the after-fermentation under the extremely difficult conditions obtaining proceeds unaided, which was formerly more or less accidental and often caused the loss of large sums of money; the use of pure yeast further renders it possible to choose such yeast races as will produce little turbidity of the wine in bottle in spite of vigorous fermentation, as they separate easily and remain clinging to the cork.

The use of selected yeast races has also proved of value in the preparation of sweet wines. Its value lies chiefly in the fact that, by the aid of a yeast which is equally vigorous to resist and to ferment in a high concentration of sugar and alcohol, wines may be produced with certainty which contain always the same amount of alcohol. A further advantage is that these wines are ready sooner, and are finer toned and preserve better than those spontaneously fermented. W. Seifert has the merit of having first made researches in practice on this subject, and of having introduced the pure culture method into this branch of wine production in the large businesses in Austro-Hungary.

The Pure Culture System in the Manufacture of Cider.—The employment of selected pure yeast in the manufacture of cider is no less important than in wine manufacture. Besides the above named, Jörgensen, Kayser and Nathan have worked specially at this subject. The procedure is the same as in the preparation of wine. The results are also very satisfactory here, and ciders prepared by means of pure wine yeasts assume a more or less vinous taste and smell.

The Pure Culture System in the Manufacture of Spirit and Pressed Yeast.—The credit of bringing the pure culture system into general recognition in spirit manufacture is due to the station in Berlin. The yeasts prepared by P. Lindner (Races I. and II., especially the latter) are employed in numerous distilleries and have given good results.

During the last few years the principles of race selection and pure culture have been applied in the preparation of a lactic acid bacterium for use in the above-mentioned industry; a mass culture from this bacterium is used for souring in order to prevent the injurious butyric acid fermentation. The first to introduce this pure culture into practice was Fr. Lafar. The use of lactic acid bacteria for souring the yeast mash has been considered a necessary evil by all the leading technologists. Recently, Wehmer has attempted to use lactic acid directly instead of the bacteria. A commercial lactic acid, not absolutely pure, can now be prepared somewhat inexpensively and he has obtained good results with it in practice as a souring material.

Finally, the pure culture system has been applied in recent times in the manufacture of pressed yeast. The Berlin station has been especially active on this subject and the use of Race V., there isolated, has spread to a great extent.

Other yeast species and races applicable to the manufactures mentioned have been isolated in the technical fermentation laboratories to be found now in every country.

SECTION III.

THE MICRO-ORGANISMS OF MOST IMPORTANCE IN THE FERMENTATION INDUSTRY.[1]

THE micro-organisms to be described now are partly useful, partly disadvantageous to the alcohol fermentation industry; their importance is therefore of widely different character. They all belong to that branch of the plant kingdom called fungi. The fungi are divided into two large groups: true fungi (*Eumycetes*) and fission fungi (*Schizomycetes*). The first of these two groups is divided into that of the algæ fungi (*Phycomycetes*) and that of the higher fungi (*Mycomycetes*).[2] Of the numerous fungi belonging to the phycomycetes only a single group comes to be considered here, namely, that of the *Zygomycetes*, and in this only the family of *Mucoraceæ*.

Among the mycomycetes we shall refer partly to the sac fungi (*Ascomycetes*), taking representatives of the four orders, the gymnoasceæ with the family of saccharomycetes, the perisporaceæ with the family of aspergilleæ, the sphæriaceæ with the family of sphærieæ, and the discomycetes with the family of pezizaceæ—partly to a large group of fungi, the imperfect fungi (*Fungi imperfecti*), of no less importance, but which cannot yet be classified;

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[1] A bibliography is given at the end of the book.

[2] We refer those wishing a more detailed description of the fungus system and the general morphology and physiology of fungi to Zopf's *Handbuch der Pilze*.

ay are in all probability only stages of development of her forms of fungi.

A review of the systematic connection of the micro-ganisms to be described here may be made by aid of the lassification given on page 173.

I.—True Fungi (Eumycetes)

is the name given to those fungi of which the vegetative organ is a mycelium. This consists of long threads which possess growing points and exhibit true branching. The first division is distinguished by this means from the second, which contains the fission fungi.

1.—*Algæ fungi* (*Phycomycetes*).

The whole mycelium consists as a rule only of one single, very much branched cell. Septa only appear under special conditions, and first appear normally when fructification begins. Endogenous spores are formed in sporangia. The only group belonging to this division which we will consider here is that of the

Zygomycetes.

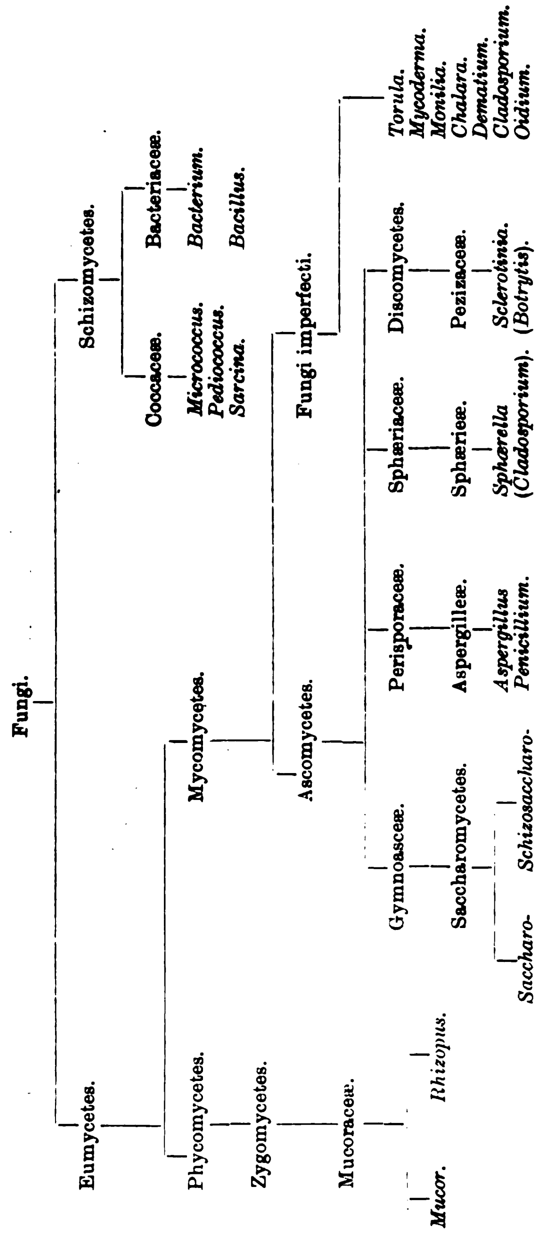
Multiplication takes place in these fungi partly by means of spores in sporangia, partly by means of the so-called zygospores, and in some forms by budding, by gemmae (chlamydospores) and by conidia.

Mucoraceæ.

The spores develop from the mass of plasma in the interior of the sporangium, a part of the plasma being left which swells by taking up water. This happens as soon as the spores are ripe, and the wall of the sporangium bursts, setting free the spores. These fungi can propagate themselves not only by means of endospores, but also by zygospores (Fig. 55, V. and VI.). The latter are produced in the following manner: Two club-shaped swellings develop on two neighbouring mycelial threads; these grow towards one another until their ends touch, which then become flat. The two flattened end membranes then coalesce. A septum is then formed in these club-shaped growths, so that an end cell—a copulation cell (Fig. 55, V., *c*)—and a suspensor (Fig. 55, V. and VI., *b*) appear. Finally the two end cells melt into one and a zygospore is thus formed (Fig. 55, VI., *a*). Some species form zygospores easily; with others it seems to be accidental; in short, the conditions of their formation are not yet known. The communications made by Bainier and others on this subject do not hold as regards those species with which the author has experimented.

A third means of propagation is possessed by some species; this consists of the so-called gemmæ or chlamydospores (Fig. 58, *a*). When the mycelium is immersed in a culture liquid containing sugar, numerous dividing walls make their appearance; short members are thus formed, which swell to a barrel shape and become highly refractive, after which their cell walls thicken. The separate members may grow into mycelium, or develop sporangium carriers at once (Fig. 58, *b*), or they may separate from one another and increase by budding like yeast cells; the so-called "spherical yeast" is thus formed (Fig. 59). The spores may also behave in this manner.

Hansen has established the following general law for fungi, that the temperature maximum for the development of the organs of propagation lies lower than the maximum for the development of the vegetative organs. This, of course, applies also to the *Mucoraceæ*. Not long ago he described two new species, *Mucor alpinus* and *Mucor neglectus*, which can both develop zygospores. He showed


Fungi.
Schizomycetes.
Coccaceæ.
Bacteriaceæ.
Micrococcus.
Pediococcus.
Sarcina.
Bacterium.
Bacillus.
Eumycetes.
Mycomycetes.
Phycomycetes.
Zygomycetes.
Mucoraceæ.
Mucor.
Rhizopus.
Ascomycetes.
Fungi imperfecti.
Gymnoasceæ.
Perisporaceæ.
Sphæriaceæ.
Discomycetes.
Saccharomycetes.
Aspergilleæ.
Sphærieæ.
Pezizaceæ.
Saccharomyces.
Schizosaccharomyces.
Aspergillus
Penicillium.
Sphærella
(Cladosporium).
Sclerotinia.
(Botrytis).
Torula.
Mycoderma.
Monilia.
Chalara.
Dematium.
Cladosporium.
Oidium.

that the temperature maximum for development of sporangia and zygospores is lower than that for development of mycelium, yeast cells and gemma formation, and that the temperature limits for sporangium and zygospore formation change with the species; thus, in *Mucor alpinus* sporangium formation has a higher temperature maximum than zygospore formation, whilst for *Mucor neglectus* the reverse is the case.

(1) *Genus: Pin Moulds, Mucor, Micheli.*

Characteristic of this order is the ball-shaped sporangium (Fig. 54, III. and IV.) which occurs at the point of the sporangium carrier which is undivided in most kinds, a "columella" separating sporangium and carrier (Fig. 54, III., *b*). This columella is formed by the more or less vesicular end of the sporangium carrier projecting into the sporangium. A crust of calcium oxalate (Fig. 54, III., *c*) is often present on the outer wall of the sporangium.

The species belonging to this order live either as saprophytes, *i.e.*, on dead animal or plant matter, or as parasites, *i.e.*, on living organisms. They may be frequently seen as a white, gray or brown felt on dung, bread, fruit, corn, malt, etc., and they also thrive in beer wort. Some species can cause dextrin to ferment, others again contain diastase.

Hansen investigated several of these species with regard to their action on the four sugars: saccharose, maltose, lactose and dextrose. It appeared that lactose is fermented by no species, and saccharose only by one single species after previous inversion, while on the other hand all the species investigated ferment dextrose and maltose. The fermentation of maltose proceeds very slowly and only forms higher percentages of alcohol after a relatively long time. Thus, for example, *Mucor Mucedo* in wort gave after fifteen days at 23° C. only 0·4 vol. per cent. alcohol; after two and three quarter months at the room temperature it had formed vol., after six months 3 vol, and after one year only 3·1 vol. per cent of alcohol.

The fermentability of the various species proved to be very varied. While, for instance, *Mucor Mucedo* did not reach 4 vol. per cent. of alcohol, *M. erectus* gave 8 vol. per cent. The facts communicated about alcohol formation in the following are from Hansen.

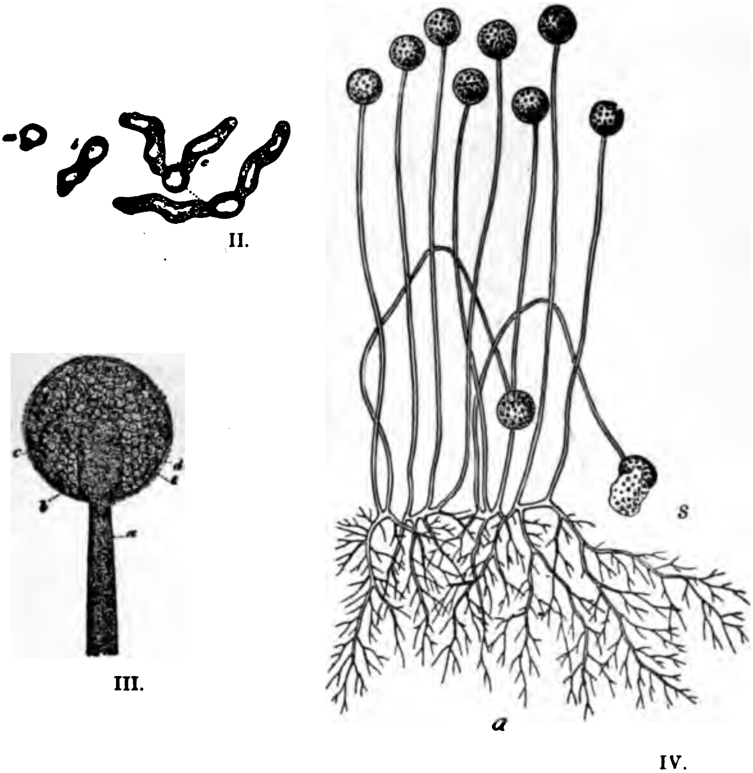
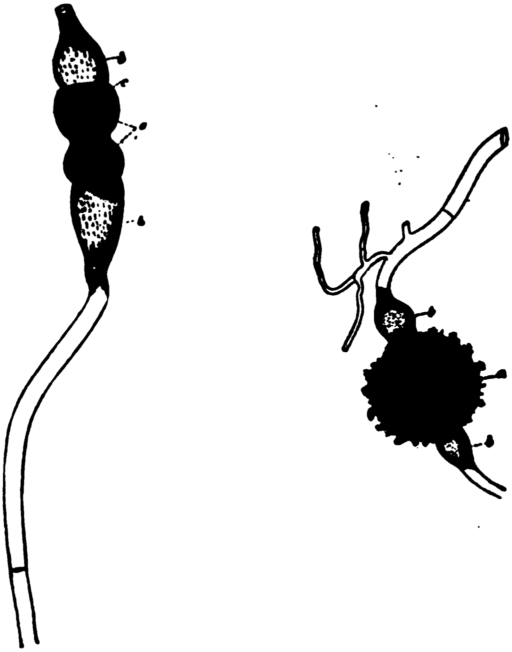


FIG. 54.—*Mucor Mucedo*, L. I., Spores. II., Germinating spores. III., Sporangium: *a*, sporangium carrier ; *b*, columella ; *c*, calcium oxalate crystals ; *d*, spores ; *e*, plasma between the spores. (After Brefeld.) IV., *a*, mycelium with sporangium carriers ; *s*, a bursting sporangium. (After Kerner.)

The powerful yeast fungi of this order show top fermentation phenomena. Emmerling found that, simultaneously with the formation of alcohol during the fermentation, glycerine and succinic acid are formed also in about the same proportion as in *Saccharomyces* fermentation.



V. VI.

FIG. 55.—*Mucor Mucedo*, L. V., *a*, Zygospore formation ; *b*, suspensors ; *c*, copulation cells. VI., A complete zygospore, *a* ; *b*, suspensors. (After Brefeld.)

The above-mentioned formation of spherical yeast and gemmæ has no connection with the formation of alcohol. Thus *M. Mucedo*, *e.g.*, yields alcohol without possessing these organs, just as the latter are found in species which have no fermenting power. They are, however, strongly developed in all species of considerable fermenting power.

In a 10 per cent. aqueous cane-sugar solution the Mucor species, like the saccharomycetes, are very tenacious of life. All the species investigated by Hansen remained alive for seven years; he proved, with regard to some species, that they were alive after more than eleven years.

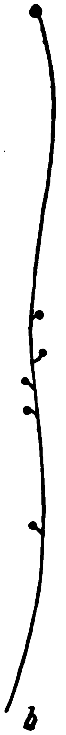


FIG. 56.—*Mucor racemosus*, Fres. A branched carrier with larger sporangium at the top and smaller ones on short side branches. $\frac{30}{1}$. (After Fresenius.)

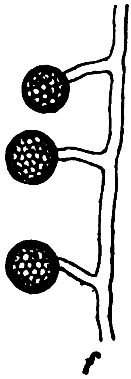


FIG. 57.—*Mucor racemosus*, Fres. Three sporangia with transparent membrane, through which the spores are seen. $\frac{240}{1}$. (After Fischer.)

They lived, dried on filter paper, for more than four years.

Mucor Mucedo, L. (Figs. 54 and 55).—The mycelium is first white, later light brown. The sporangium carriers are often about 10 cm. long; the sporangium (Fig. 54, III.

and IV.) is spherical, large, with a diameter of 100 to 150 μ; it is first yellow, later grey and nearly black. The columella (Fig. 54, III., *b*) is a short cylinder with a dome-shaped end. The spores (Fig. 54, I.) are ellipsoidal, 7 to 12 μ long and 4 to 7 μ thick, with a colourless membrane and yellowish contents. The zygospore (Fig. 55, VI.), which was found on dung, is large (90 to 200 μ), spherical, brownish-

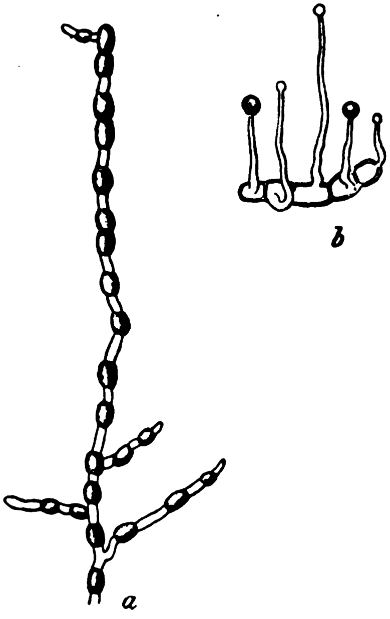


FIG. 58.—*Mucor racemosus*, Fres. *a*, Part of a mycelium, the contents of which have contracted to numerous gemmæ. $\frac{120}{1}$. *b*, Five gemmæ together, which have germinated into small undivided sporangium carriers. $\frac{200}{1}$. (After Brefeld.)

black and with wart-like excrescences. Under special conditions (lowering of temperature, impaired nourishment or parasitical attacks) the sporangium carriers have the power of branching; the branches then often bear sporangia without columellæ, so-called sporangioles. This species does not exhibit gemma formation.

Mucor Mucedo liquefies wort gelatine when it grows on this medium. It forms in wort, after one year at the room temperature, 3·1 vol. per cent. of alcohol, and it appears to have then reached its maximum. In a 5 per cent. solution of maltose in yeast water it exhibited a feeble but distinct alcoholic fermentation. In a 10 per cent. solution of dextrose in yeast water it formed 0·8 vol. per cent. of alcohol after one and a half month.

The fungus is extraordinarily widely distributed in nature, and is found everywhere on manure, decomposing vegetable matter and in soil.

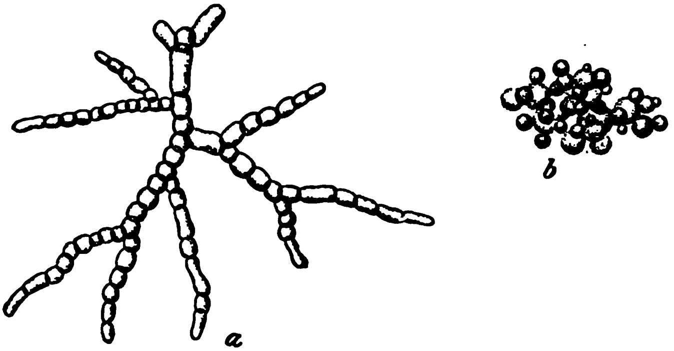


FIG. 59.—*Mucor racemosus*, Fres. *a*, A piece of mycelium immersed in sugar solution, separating into spherical yeast; *b*, Spherical yeast multiplying by budding. $\frac{120}{1}$. (After Brefeld.)

Mucor racemosus, Fresenius (Figs. 56, 57, 58 and 59).—The sporangium carriers (Figs. 56 and 57) are, as a rule, branched, 2 to 3 cm. high; the sporangia (Fig. 57) spherical, brownish and 30 to 40 μ in diameter. The spores are ellipsoidal or spherical, 5 to 8 μ long and 3 to 5 μ thick. The columella is pear-shaped. Zygospores occur very seldom, and are spherical, 70 to 84 μ thick, yellowish and provided with brown, lumpy or ridged thickenings. This species displays a very abundant gemma formation (Fig. 58).

Hansen found the following temperature limits: In wort and on wort-agar-gelatine, the maximum temperature for the development of the mycelium is 32° to 33° C., of yeast cells and gemmæ 32° C., and the minimum temperature for the mycelium and gemmæ ½° C. The temperature limits for the development of sporangia on wort-agar-gelatine are 31° to 32° C. and 3° to ½° C.

When *Mucor racemosus* is seeded on wort gelatine, its felty appearance quickly changes, the surface becoming clear and the gelatine liquefying. At the ordinary room temperature after fourteen days, it forms 1·3 vol. per cent. of alcohol in wort, after one year 7·0 vol. per cent. It ferments maltose. At 25° C. it forms, in yeast water containing 10 per cent. of dextrose, 2·6 vol. per cent. of alcohol after one and a half month, and at 25° C., 2·3 vol. per cent. of alcohol in yeast water containing 10 per cent. of cane sugar after one and a half month. This fungus is the only one of the hitherto investigated *Mucor* species which generates invertase, and, in consequence, is able to transform cane sugar into invert sugar and to ferment the latter.

This fungus is widely distributed and occurs particularly on plums.

Mucor erectus, Bainier, is closely related to the foregoing species, and was formerly confused with it. It does not, however, contain invertase; on the other hand, it can transform starch into a reducing sugar.

When it is sown on wort gelatine, a growth develops which is morphologically similar to *M. racemosus*. At the ordinary room temperature after two and a half months it forms 8 vol. per cent. of alcohol in wort, and at 25° C. 7 vol. per cent. of alcohol after the same period. In a 10 per cent. solution of dextrose in yeast water at 25° C. it forms 3·5 vol. per cent. of alcohol in fifteen days. In several respects it thus excels brewery yeast, being able to produce a higher unt of alcohol in wort; but, as regards rapidity of fer- tation, it is inferior.

Mucor oryzæ, Went and Prinsen Geerligs. — The elium has rhizoid-like offshoots. Gemmæ (chlamydo- es) are the only known organs of multiplication. s species is found in "raggi," *i.e.*, in a mixture of rice various organisms which is used in the manufacture of ck in Java. It transforms the rice starch into dextrose, ch is then fermented by the yeasts present. Its gemmæ not produce alcohol. It may possibly be identical with

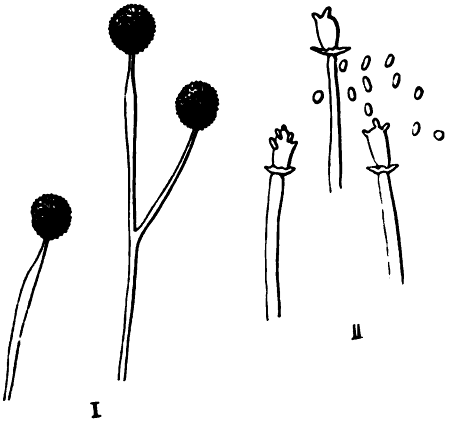


FIG. 60.—*Mucor spinosus*, van Tieghem. I., Sporangium carrier with sporangia. II., Sporanguim carrier with columella and loose spores. (After Gayon.)

the *Rhizopus oryzæ* to be described later, which is also found in "raggi".

A species related to the above and employed in the same manner is

Mucor (Amylomyces) Rouxii, Calmette, which is propagated by chlamydospores and also by means of sporangium fructification; this latter fact was discovered by Wehmer. The growths are grey, light yellow to light yellowish-brown on agar-agar, but on rice they are orangeyellow. The sporangia are colourless to yellowish, spherical, about 50 μ in diameter, smooth and translucent. The columella is spherical, smooth and colourless, and the spores are long shaped ($5 \times 2{\cdot}8\ \mu$), seldom round, colourless, smooth and shining. The best growing temperature for the fungus is 30° to 40° C. It is found on rice husks, and is made from these into "Chinese yeast," which is a common article of commerce in East Asia, and which contains, besides this species, various saccharomycetes, aspergillæ and bacteria. Its function consists, like that of the above species, in changing the rice starch into sugar, and thus making the latter capable of fermentation. This fungus can ferment sugar itself, like most other species of *Mucor*, but the process is best performed by saccharomycetes. The species has begun to be used, of late years, in spirit manufacture, but, as it seems, without real success.

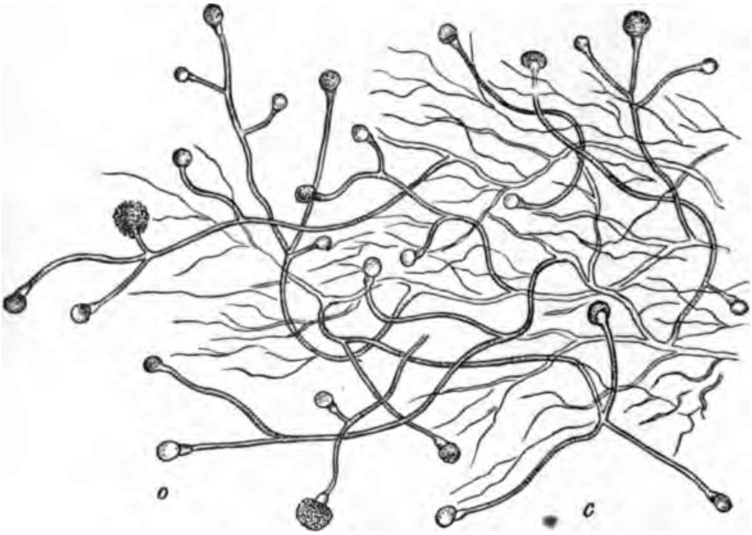


FIG. 61.—*Mucor corymbifer*, Cohn. Mycelium with underlying branched carriers. The sporangia at *o* have burst and the columella together with the expanded end of the carrier form a pear-shaped body. $\frac{270}{1}$. (After Lichtheim.)

Mucor spinosus, van Tieghem (Fig. 60), is dis-guished by the thorny, irregular growths frequently nd on the columella (Fig. 60, II.); these sometimes pear only as bacterium-shaped growths, or they may entirely absent. If the fungus is sown on wort gelatine, e latter becomes covered with a brown felt, and is quefied. Gemmæ are formed.

The fungus forms 5·5 vol. per cent. of alcohol in wort at 2° C. in a year. In a maltose solution it soon shows signs of fermentation, and forms 3·4 vol. per cent. of alcohol in eight months. In yeast water, to which 10 per cent. of dextrose has been added, it produces 2 vol. per cent. of alcohol in sixteen days at 25° C.

Mucor alternans, van Tieghem, according to Gayon and Dubourg's researches, is able to change dextrin and starch into sugars, and to ferment these substances. It forms alcohol up to 4·2 vol. per cent.

Mucor corymbifer, Cohn (Fig. 61), differs considerably in appearance from the forms hitherto described. The mycelium is white, later on light grey, and forms a very thick felt, the separate mycelium threads being very long. The sporangium carriers do not grow out straight, but form decumbent umbellate raceme-like branches, the ends carrying from one to twelve umbellate sporangia; below the end-umbel these fruit carriers develop, in addition, a number of single, short-stalked, smaller, racemose and to some extent dwarfed sporangia. The sporangium carriers are expanded below the sporangia. The latter are colourless, pear-shaped and 10 to 70 μ in diameter. The columella is conical, broad at the top and often warty and brownish. The spores are very small and elliptical, being 3 μ long and 2 μ broad. The optimum temperature is remarkably high (37° C.). The fungus is pathogenic to man, and has been found, for example, in the eye. Wort gelatine is not liquefied by this fungus at ordinary temperature even after three months.

(2) *Genus : Rhizopus, Ehrenberg.*

This differs from the first genus in that the mycelium threads develop stolon-like side branches (Fig. 62, *a*), which grow archwise through the air, until their ends come again into contact with the substratum, when peculiar adherent organs, rhizoids, are developed. The sporangium carriers appear where these rhizoids are produced.

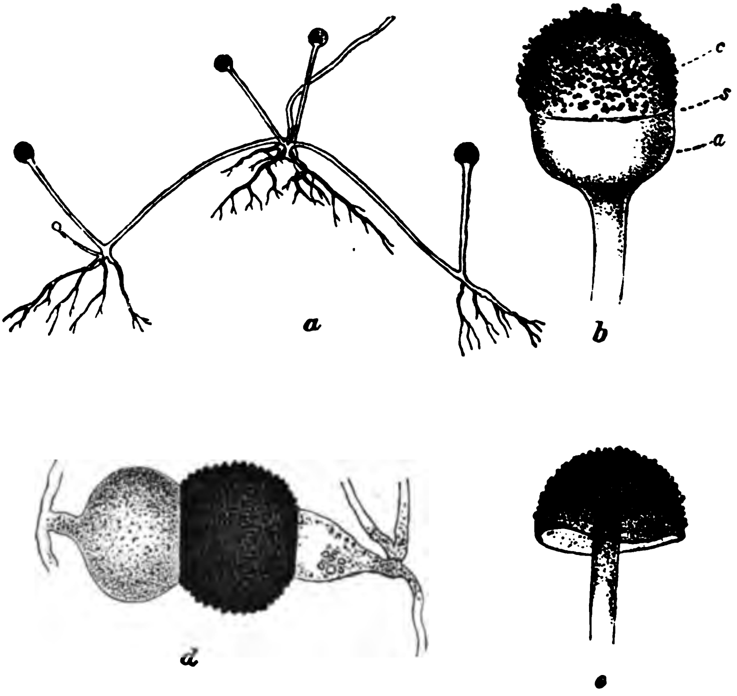


FIG. 62.—*Rhizopus nigricans*, Ehrenberg. *a*, Runners or stolons and sporangium carriers, there being usually more of the latter (3 to 5) than are shown in the figure. About $\frac{8}{1}$. (After De Bary.) *b*, A columella (*c*) the lower part of which (*a*) is free ; the sporangium wall grew from *s*. $\frac{100}{1}$. (After Fischer.) *c*, A collapsed mushroom-like columella covered, like the previous one, with spores. $\frac{100}{1}$. (After Fischer.) *d*, Ripe warty zygospore. $\frac{90}{1}$. (After De Bary.)

Rhizopus nigricans, Ehrenberg (*Mucor stolonifer*, Ehrenberg), is represented in Fig. 62. Two to five sporangium carriers are found together; they are about 2 to 4 mm. long, and carry a globular blackish-brown sporangium, which encloses almost the upper half of the columella (Fig. 62, *b*), leaving the lower part free. The columella is well-developed and dome-shaped ; after the spores have been set free it collapses into an umbrella or mushroom shape (Fig. 62, *c*). The spores are slightly angular and provided with ridged thickenings, being about 9 to 17 μ in diameter, and of a greyish-brown colour. Zygospore fructification has been observed (Fig. 62, *d*) on unripe gooseberries and on earth-nut cake. The suspensors are thick-bodied, whilst the zygospore, which is covered with hemispherical warts, is barrel-shaped and has a diameter of from 170 to 220 μ.

This species sometimes occurs in large quantity on broken grains, and it is also productive of decay in many fruits, especially apples. J. Behrens found that this fungus secretes a protoplasm-poison, *i.e.*, a substance which has a poisonous action on the protoplasm of the living fruit cell. It can also convert starch into sugar.

Rhizopus oryzæ, Went and Prinsen Geerligs.—The sporangia are blackish-brown with pear-shaped columellæ, and frequently a rim remains after the bursting of the sporangium wall. The size is variable, the length often being about 175 μ, and the breadth about 100 μ. The spores themselves are somewhat angular, light grey, 7 μ long and 5 μ broad. Gemmæ are produced in abundance, whilst peculiar wreath-like branchings occur on the aerial mycelia.

It changes rice starch into dextrose and is a constituent of the "raggi" mentioned above, employed in the manufacture of arrack in Java. Its probable relationship to *Mucor oryzae* has been already stated.

II.—THE HIGHER FUNGI (MYCOMYCETES).

The mycelium is divided by septa.

A.—SAC FUNGI (ASCOMYCETES).

The fungi belonging to this division form endospores in a sporangium which is called an ascus or sac. Many such asci may be enclosed in a distinct outer envelope.

Order I.—Gymnoasceæ.

These are the simplest of the ascomycetes. The asci have no outer coating.

TRUE YEAST FUNGI (SACCHAROMYCETES).

General.

To this family belong all true alcoholic yeast fungi, on the activity of which the alcoholic fermentation industry depends; to it also belong some of the most formidable enemies with which this industry has to contend.

1.—*The Saccharomycetes Distinct Fungi.*

Since the year 1837, when it became evident that yeast is a vegetable growth, it has been asserted at different times by various authorities that yeast is not an independent organism, but only a separate stage of development of some higher fungus. Experimental confirmation of this was attempted, and, as the methods of that time were very imperfect, some very remarkable results were obtained. The mould fungi, especially, were accepted as the probable parent growths; this assumption was apparently strengthened by the discovery of the formation of yeast-like cells in *Mucor*, for here were budding cells which, like real yeast, were capable of forming alcohol. Sometimes it was believed to have been proved that *Penicillium* or *Mucor* were the parent forms, sometimes *Ustilago*, *Aspergillus*, *Sterigmatocystis*, *Dematium*, etc. Claims for the latter, especially, have been put forward quite recently.

Whereas formerly only alcohol-forming yeast fungi were understood under the denomination "yeast," this term was extended later to include all budding fungi. After it became known that *Ustilago*, the smut found on grain, forms budding cells, it was asserted that the yeast cells of *Ustilago*, and the alcoholic fermentation fungi of practice, were morphologically identical. It was again inferred from this that yeast is derived from *Ustilago*. Although this opinion was never proved, it was yet strong enough to cause confusion of ideas. Even now the word "yeast" is frequently used in text-books to designate not only true saccharomycetes, but also all budding fungi.

There exists at the present time, meanwhile, no proof at all that saccharomycetes are a stage of development of other fungi. On the contrary, we must regard them as ascomycetes equally as independent as, *e.g.*, the exoasceæ, the independence of which no one has doubted, and to which group they are closely connected, appearing in the same forms (budding cells, endospores and mycelium) as the exoasceæ, and in these forms only.

2.—*Structure and Shape of Yeast Cells.*

A *Saccharomyces* growth always consists of budding cells, under certain conditions also of asci with endospores and of mycelium.

The budding cell consists of a membrane enclosing a mass of protoplasm in which there is a cell nucleus.

The Cell Contents.—The cell nucleus, the existence of which was proved by Schmitz in 1879, is, as a rule, a spherical body; Hansen has, however, in some cases observed flat nuclei. In each yeast cell there is only one nucleus. To see this it is usually necessary to fix, harden and stain the preparation (see p. 89). Only in exceptional cases can they be seen without this preliminary treatment. The cell nucleus has been studied in recent times by Jannsens, Leblanc and Wager. Essential points with regard to its structure and function have not yet been cleared up.

According to Jannsens and Leblanc the protoplasm of the yeast cell forms a fine network; during fermentation its appearance changes: vacuoles appear, the protoplasm becomes granular, and fat and oil particles may be formed. By vacuoles are understood cavities in the cells, which are filled with cell sap. A highly refractive body, the vacuole-granule, is usually seen in vacuoles, which is in constant motion, the so-called Brownian molecular movement. The number of these granules is usually one to three; only in exceptional cases are there more than three or none at all. According to Küster these bodies are decomposition products derived from the plasma; they form a plastic, semi-fluid mass. Using a weak aqueous solution of neutral red (1 to 5,000 or 1 to 10,000) they become intensely red in a few minutes, if the material is suitable, all other parts of the cell remaining colourless. Vacuole-granules coloured in this manner give up the colouring matter in a sugar solution, the latter gradually becoming red.

In the cell protoplasm may often be seen numerous granules usually of angular shape. They are easily stained by aniline dyes, especially methyl green (Casagrandi). They are soluble in alcohol, ether, chloroform, chloroform and ether, caustic potash, caustic soda, petroleum ether, etc.; sometimes they require several days to dissolve. According to Will they are of a fatty nature; he has further observed that when the oily substance had been removed by treatment with absolute alcohol, small faint bubbles appeared in the place of the granules, in the interior of which a network could be seen.

If absolute alcohol is added to a preparation the cells may be seen to shrink up, and are soon killed. In general cells are distinguished from living ones, as we saw in on II., by the greater ease with which they take up uring matter.

he Cell Wall and its Gelatinous Formation.—The brane of the yeast cell is very thin in young individuals; in old cells, and in such as live under unfavourable conditions of nutrition, it may become tolerably thick, *e.g.*, several micromillimetres. It can be shown distinctly by means of various reagents (*e.g.*, dilute acid and alkalis). According to Will's and Casagrandi's experiments the membrane con-

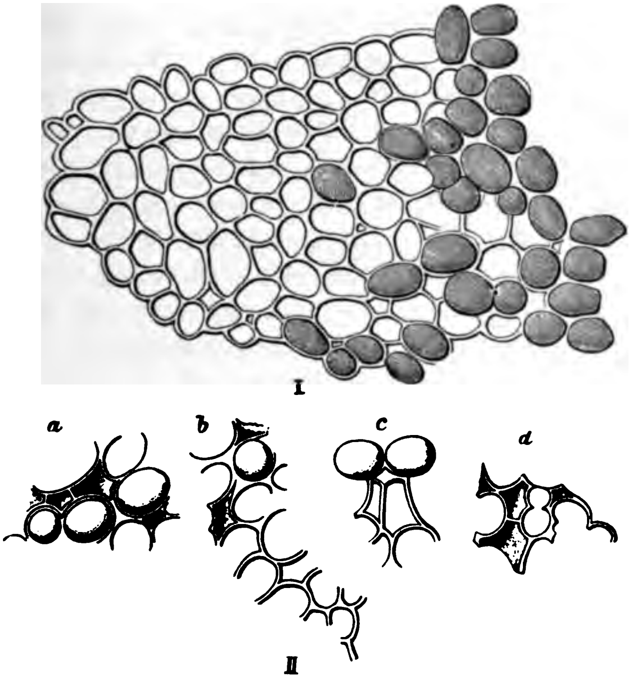


FIG. 63.—Gelatinous network. I., Network formation and yeast cells which are stained with methyl violet; most of the cells have disappeared; some (on the right) still lie in the meshes. II., It may be seen in *a*, *b* and *d* that the network forms complete walls; there are in *a* 3 cells, in *b* 1, and in *c* 2 cells lying in the meshes. (From Hansen's original drawing.)

sists of two or more layers; this may be shown by treating the cells for a long time (days to weeks) with a 1 per cent. solution of osmic acid. The membrane dissolves easily in concentrated chromic acid, more slowly in concentrated sulphuric acid (Casagrandi).

The membrane of the cell gives off, under certain conditions, a mucilage which takes part in the formation of the gelatinous network described by Hansen (Fig. 63). After hardening an ordinary microscope preparation it shows itself in the form of strands and plates between which the cells are enclosed. The granulations originally present between the cells may be taken up into the substance of the network, which may be stained by this means. This formation may bė readily obtained if a lump of thick yeast, as it usually occurs in breweries, is placed in a glass, covered, and put away for a short time until partial drying takes place. It usually occurs also in spore cultures on gypsum blocks, on gelatine and in yeast ring formations.

Will considers the network, which appears, *e.g.*, during the drying up of beer yeast, to be different from that which occurs in film formations and in the yeast ring. In his opinion a gelatinisation of the cell membrane possibly takes part in bringing about the former, the albumen mixed up with the yeast, however, playing the chief part. The networks formed in the film and in the yeast ring may also, according to him, be of different constitution, since some forms are found which give the albumen reaction and some which do not. It is very difficult to distinguish here what, in the network formation, originates in the cell wall itself, what in the cell contents, and what in the surrounding medium; this is perhaps a problem which does not as yet admit of a solution.

Shape of the Cells.—Budding cells occur especially in or upon nutrient liquids, but are also found on solid substrata.

Their shape is exceedingly varied; they are spherical, egg-shaped, oval, sausage-shaped, filament, dumb-bell, and lemon-shaped, etc., besides occurring now and then in cultures in quite irregular and abnormal forms. It is a noteworthy fact that no definite cell shape is absolutely peculiar to one species; it is true that the majority of the cells of a species, under certain conditions of culture, occur in a certain shape; but if these conditions are altered, the shape will alter also. A *Saccharomyces* species can seldom or never be determined by microscopic observation alone. Hansen clearly demonstrated this multiplicity of form of the species and the defectiveness of the classification which had been employed before his time. Simultaneously he showed that, under definite conditions of culture, the shape of the cells affords a good group—characteristic for species. (Compare p. 233, " Variation ".)

3.—*Modes of Propagation.*

Budding.—Vegetative increase (the formation of new vegetative cells) takes place in all true saccharomycetes by means of budding. This takes place by a small outgrowth forming on the mother cell (Fig. 64), and gradually increasing in size.

The new cell may separate from the mother cell, or may remain connected with it; in the latter case large colonies of buds are formed (Fig. 64). In the genus *Schizosaccharomyces* vegetative increase takes place not by budding, but by splitting off. Near the middle of the mother cell is formed a septum which splits up and thus sets the new cell free.

The budding of a single cell was studied by Mitscherlich in 1843; he distributed a little yeast in beer wort, so as to obtain one or two cells in an ordinary sealed up preparation, and gave a drawing of the various generations (Fig. 64). Under these circumstances thirteen hours passed before the seeded cell had formed a new ce size. When the preparation was three days o of descendants of the original cell had increas time. Kützing proceeded in the same way. S later Pasteur also repeated this experiment, l difference that, instead of placing wort on the used grape juice. He then found that each yeast cells present had formed three cells in the two hours.

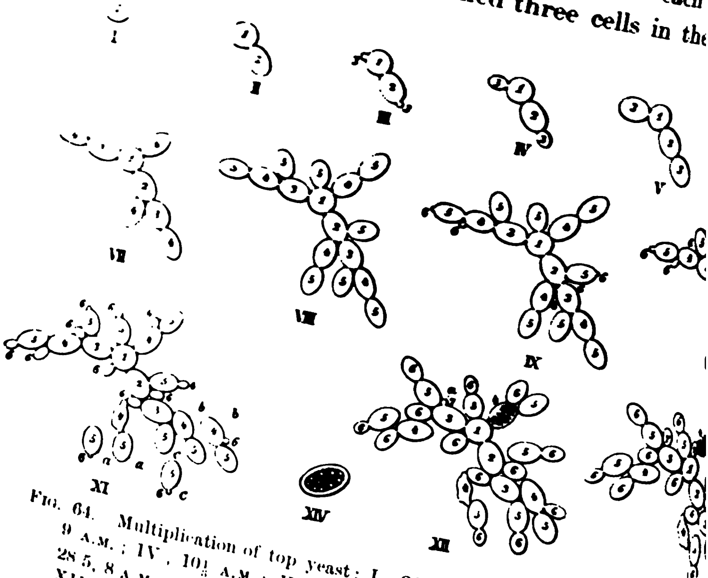


FIG. 64. Multiplication of top yeast: I., 26/5, 7 P.M.; II., 27/5, 8 A.M.; III. 9 A.M.; IV., 10½ A.M.; V., 12 NOON; VI., 3½ P.M.; VII., 8 P.M.; VIII., 28/5, 8 A.M.; IX., 10 A.M.; X., 11 A.M.; XI., 1 P.M.; XII., 29/5, 8 P.M.; XIII., 30/5; XIV., 2/6, 12 o'clock. (After Mitscherlich.)

In studying the budding of yeast by the above method Ranvier's moist chambers (see pp. 69 and 93) are the most convenient to employ. Further information on this subject may be obtained by studying a series of drawings from life history, such as those from Hansen given in Figs. 82, 83, 84, 118 and 119, in which data with regard to time are furnished.

Further thorough investigations of yeast multiplication under various conditions have been made by Rasm. Pedersen, and afterwards by Hansen, Hayduck and others. The method employed in these experiments consisted in counting the yeast cells by means of counting chambers. Rasm. Pedersen carried out his experiments with brewery bottom yeast which had been cultivated in unhopped wort. The times required to complete the generation of a cell were, for different temperatures, as follows: 20 hrs. at 4° C., 10·5 hrs. at 13·5° C., 6·5 hrs. at 23° C., 5·8 hrs. at 28° C., and 9 hrs. at 34° C.

As one might expect, the various species and races increase at different rates. This was first proved by Hansen's comparative experiments on *Saccharomyces apiculatus* and some bottom yeast species. These will be more fully described later on. In order to characterise species in this way it would be necessary to carry out an extremely large number of similar experiments; this has not yet been done.

The vigour with which vegetative increase proceeds depends of course on the conditions under which the species are cultivated, and the terms energy of multiplication and power of multiplication under certain conditions of cultivation are employed. By energy of multiplication is understood the number of cells produced by one cell in a certain time, and power of multiplication denotes the absolute number of cells which one cell is capable of generating.

Aëration of the culture liquid must be mentioned as one of the foremost factors in accelerating vegetative increase. Temperature also plays an important part, and as important, of course, is the chemical composition of the culture liquid. Examples of excellent culture liquids are those in general use in the fermentation industry, *viz.*, wort, must, etc. The lowest temperature at which budding is observed with saccharomycetes lies near 0° and the highest is about 47° C.; but species differ also in this particular. For example, *Sacch. Pastorianus I.* and *Sacch. Pastorianus II.* have considerably lower minimum temperatures than *Sacch. cerevisiæ I.* The maximum temperature for *Sacch. Pastorianus I.* is about 34° C., whilst *Sacch. cerevisiæ I.* exhibits a vigorous increase at 40° C. and *Sacch. Marxianus* even at about 47° C. The above holds for those cases in which the culture liquid is the ordinary hopped wort (14 per cent. Balling) as used in bottom fermentation breweries. Although the opinion obtained formerly that top yeasts were able to produce buds at higher temperatures than bottom yeasts, this is far from being generally the case. There are top yeasts in which budding ceases at a much lower temperature than that for certain bottom yeasts, *e.g.*, the top yeast *Sacch. Pastorianus III.* and the bottom yeast *Sacch. ellipsoideus I.*

When a yeast cell is seeded in a nutrient liquid, it sinks to the bottom and forms a colony, as we have seen in the foregoing; gradually the cells separate from one another and form new colonies. The development of carbonic acid gas during fermentation causes the cells to be carried about in the liquid. If it is a top yeast, the cells rise in large quantities to the surface and may form there a thick layer of yeast on the froth. When fermentation is finished, the yeast again sinks to the bottom and there forms a more or less solid sedimentary yeast. Good brewery yeast usually gives a dough-like deposit which lies close on the bottom, while on the other hand many wild yeasts give a cheese-like sedimentary yeast which either lies on the bottom in crumbs and lumps or is partially distributed throughout the liquid in a finely divided state. By a certain treatment several species may be induced to form cheese yeast. Single cells may, however, after fermentation is over, remain on the surface of the liquid and form a film there just as some develop a yeast ring on the wall of the vessel along the edge of the surface of the liquid.

Film formation at the surface of fermenting liquids is a phenomenon of widespread character; it occurs in many different microscopic fungoid forms. As pure cultures were not in use long ago, the observations then made often related to such film growths as were formed in common by several different species, saccharomycetes and non-saccharomycetes. The exact study of this subject was begun by Hansen, and it was chiefly with the following six species, to be described later, that he carried out his experiments: *Sacch. cerevisiæ I.*, *Sacch. Pastorianus I.*, *II.* and *III.*, and *Sacch. ellipsoideus I.* and *II.* The result of his investigations was as follows: In order that a *Saccharomyces* growth may form a film, the culture must be allowed to remain at rest for a long time with an abundant supply of air, the temperature being tolerably high. Now there are a few species, *e.g.*, *Sacch. anomalus* and *Sacch. membranæfaciens*, which form a film at once; the latter, however, is the usual form of growth in these individuals; sedimentary yeast is seldom or never formed directly by them, but only by the film cells sinking. These films are also distinguished from those of the typical saccharomycetes by their dull appearance, air being present between the cells as in a *Mycoderma* film: films of typical saccharomycetes are, on the other hand, slimy. The latter will be treated in what follows.

The microscopic appearance of film cells belonging to the same species varies for different temperatures; the limiting temperatures of film formation as well as the time of its appearance for different species at the same temperature vary also. Thus not only are details given about the most important conditions for the appearance of the film form, but also new characteristics of the species.

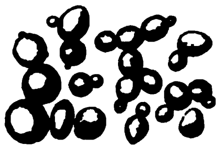


FIG. 65.—*Saccharomyces cerevisiæ I.*, Hansen. Sediment yeast. $\frac{400}{1}$. (After Hansen.)

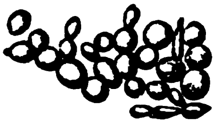


FIG. 66.—*Saccharomyces cerevisiæ I.*, Hansen. Film growth at 15—6° C. $\frac{400}{1}$. (After Hansen.)

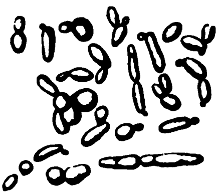


FIG. 67.—*Saccharomyces Pastorianus I.*, Hansen. Sediment yeast. $\frac{400}{1}$. (After Hansen.)

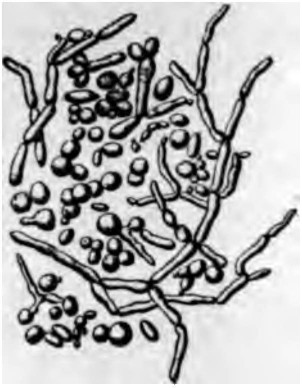


FIG. 68.—*Saccharomyces Pastorianus I.*, Hansen. Film growth at 15—3° C. $\frac{400}{1}$. (After Holm in Hansen's treatise.)

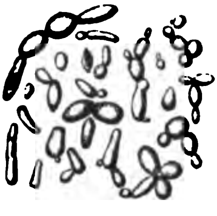


FIG. 69.—*Saccharomyces Pastorianus II.*, Hansen. Sediment yeast. $\frac{600}{1}$. (After Hansen.)

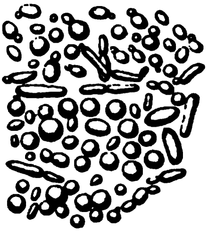


FIG. 70.—*Saccharomyces Pastorianus II.*, Hansen. Film growth at 15—3° C. $\frac{600}{1}$. (After Holm in Hansen's treatise.)

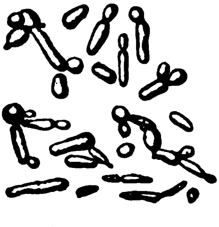
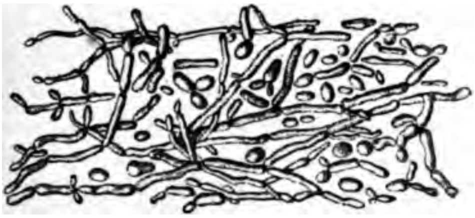


FIG. 71.—*Saccharomyces Pastorianus III.*, Hansen. Sediment yeast. $\frac{600}{1}$. (After Hansen.)



G. 72.—*Saccharomyces Pastorianus III.*, Hansen. Film growth at 15—3° C. $\frac{500}{1}$. (After Hansen.)

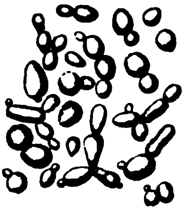


FIG. 73.—*Saccharomyces ellipsoideus I.*, Hansen. Sediment yeast. $\frac{500}{1}$. (After Hansen.)

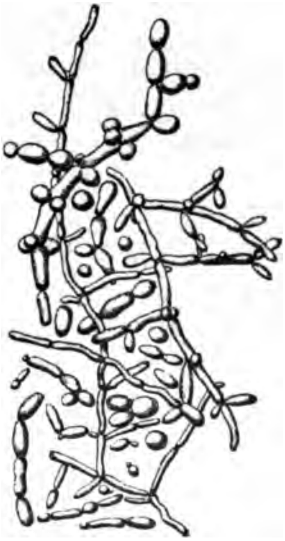


FIG. 74.—*Saccharomyces ellipsoideus I.*, Hansen. Film growth at 15—13° C. $\frac{500}{1}$. (After Holm in Hansen's treatise.)

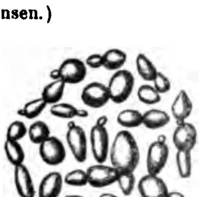


FIG. 75.—*Saccharomyces ellipsoideus II.*, Hansen. Sediment yeast. $\frac{500}{1}$. (After Hansen.)

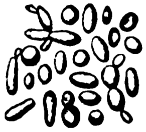


FIG. 76.—*Saccharomyces ellipsoideus II.*, Hansen. Film growth at 28—3° C. $\frac{500}{1}$. (After Hansen.

With regard to the microscopic appearance of the film cells, these are, as a rule, very long shaped in the older growths, and the cells tend to assume irregular forms; now and then they also develop a mycelium. Spores are found in the film cells as an exception, namely, in that species of film formation called the yeast ring. The latter is formed, as mentioned above, along the edge of the liquid surface on the wall of the flask and is very marked in certain species.

For instances of the varied appearance of the film cells in different species as well as when compared with bottom yeast, see figures 65 to 76.

The difference in the film cells of these species is most noticeable at 13° to 15° C.

The highest and lowest temperatures at which film formation has been observed by Hansen in the different species were given by him in 1886 in the above-mentioned treatise. They are as follows :—

Sacch. cerevisiæ I. : 33° to 34° C. and 6° to 7° C.

Sacch. Pastorianus I., II. and *III.* : 26° to 28° C. and 3° to 5° C.

Sacch. ellipsoideus I. : 33° to 34° C. and 6° to 7° C.

Sacch. ellipsoideus II. : 36° to 38° C. and 3° to 5° C.

As regards the time required for the development of the film, *Sacch. ellipsoideus II.* is especially noticeable, this species developing a very vigorous film in about ten days —often earlier—at 22° to 23° C. The other five species require a much longer time.

Will has shown that the different generations of cells formed in the film exhibit dissimilarities. His results, however, cannot be further referred to in a limited book like the present. We therefore refer those who desire to study these conditions more particularly to his papers (1895 and 1899) mentioned in the literature review for this section. With regard to the limiting temperatures of film formation for four of the species of beer bottom yeasts examined by him, a minimum temperature was

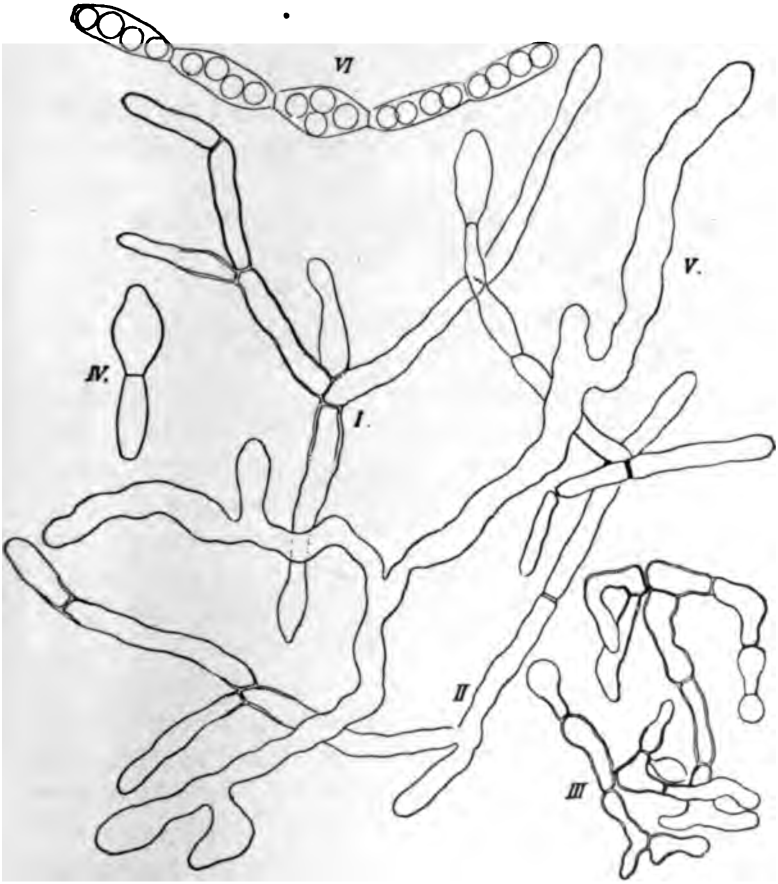


FIG. 77.—*Saccharomyces Ludwigii*, Hansen. Mycelium and spore formation from very old cultures in cherry juice and yeast water respectively. I. to IV., Mycelia or fragments of such with broad thick septa. V., An irregular, branched small mycelium completely devoid of septa. VI., Mycelium threads, also with broad septa; in each cell (ascus) 4 spores. $\frac{1000}{1}$. (After Hansen.)

found at 4° to 7° C. for two, and at 7° to 10° C. for the other two, while he found the maximum temperature for three species to be near 30° C., and, for the fourth, 28° C.

When a growth has formed a film, special chemical changes take place in the nutrient liquid. Thus wort usually assumes a light yellow colour under these conditions, and develops a disagreeable smell and taste. The formation of the film, however, is not always to blame for this, since beer which has stood for a long time, and on which no film has appeared, may likewise assume the above properties. According to Raymann and Kruis, the film cells, if allowed to remain for a long time, are capable of converting the alcohol formed in the culture liquid into carbonic acid and water by oxidation.

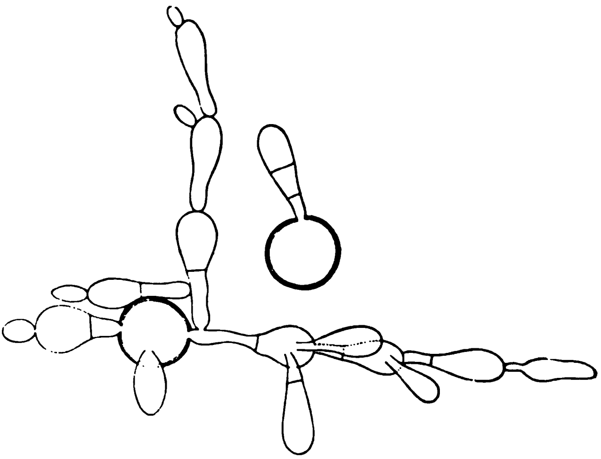


FIG. 78. - Resting Cells Germinating. (After Will.)

Hansen showed that, under certain conditions, saccharomycetes can also develop a mycelium provided with septa. He observed such an one first in *Sacch. Marxianus* and *Sacch. Ludwigii* (Fig. 77), and afterwards in some other species. It occurs chiefly in old films and in old cultures on solid nutrient material. A mycelium of this kind then frequently shows forms similar to *Dematium* or *Monilia*.

As an example of the influence of temperature on the above, an observation made by Hansen may be mentioned here, of a culture of Carlsberg bottom yeast No. 1, which had been for a long time in a saccharose solution, and which gives a mycelium on cultivation in wort at a low temperature, but not at a high one. Further, all possible transitions in cell shape can be found from round cells to colonies with branched mycelia, consisting of very elongated cells.

Of late years P. Lindner, Will and others have also observed such mycelium forms in various typical saccharomycetes. In addition the resting cells described by Will sometimes form, when germinating, mycelium-like colonies, the cells of which are often furnished with septa (Fig. 78).

These resting cells are to be found both in film and yeast ring formations. They have a strong thickened membrane which consists of two, sometimes several, layers (this can be shown by treatment with hydrochloric acid), and are rich in glycogen and oil globules. Will says with respect to the latter that they are soluble in alcohol and become grayish-green, later brownish-black by the addition of concentrated sulphuric acid, in contradistinction to the fat globules of sediment yeast cells which are difficultly soluble in alcohol and are not coloured by concentrated sulphuric acid. In cultures in which all other cells have died these resting or "durative" cells may still be found living. The more unfavourable the composition of the culture liquid is for the multiplication of yeast the quicker are cells turned into resting cells. When these germinate, the fat globules collect in the neighbourhood of the point at which the daughter cell is formed, the quantity of fat dwindling away as germination proceeds. A specially characteristic mode of germination consists in the sprouting of club-like or sausage-shaped cells from the resting cells, septa then appearing in the former. Cells with an ordinary thin wall may also keep alive a very long time and thus appear as resting cells.

Colonies formed from saccharomycetes on solid nutrient substrata also present some marks in their appearance which serve to distinguish between species, but by no means in every case, and here also we have to reckon on variations. Hansen in his first communication on the six species of *Saccharomyces* already mentioned, drew attention to the fact that differences exist among them in this respect which may be brought about by the culture medium and by temperature. He found, for instance, that *Sacch. ellipsoideus I.*, cultivated on wort gelatine at 25° C., is very different from the other five species, the surface of the colonies assuming a net-like structure ; further, that *Sacch. Pas-*

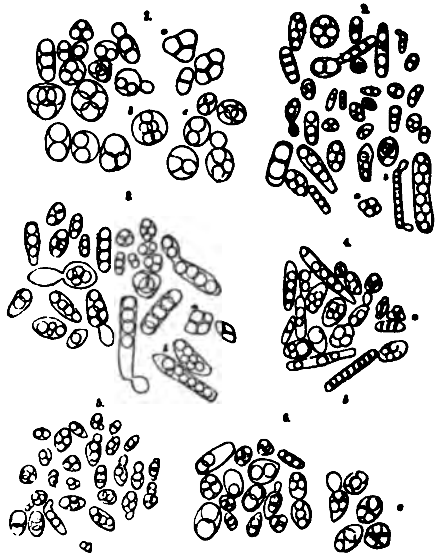


FIG. 79.—Saccharomycetes which form ascospores. 1. *Sacch. cerevisiæ I.* 2. *Sacch. Pastorianus I.* 3. *Sacch. Pastorianus II.* 4. *Sacch. Pastorianus III.* 5. *Sacch. ellipsoideus I.* 6. *Sacch. ellipsoideus II.* *a*, cells with septa ; *b*, cells with more than normal number of spores ; *c*, cells with distinct indications of spore formation. About $\frac{500}{1}$. (After Hansen.)

torianus II. in streak cultures in yeast water gelatine at 15° C. develops, in sixteen days, colonies with smooth edges, whereas the latter are hairy in *Sacch. Pastorianus III.*, under the same conditions of cultivation. Aderhold, Lindner and others have made subsequent communications on differences among the species in the above respect. For this purpose Lindner sows drops of yeast on nutrient gelatine and thus obtains the so-called giant colonies.

Spore Formation.—Besides vegetative increase by budding, saccharomycetes, like all other ascomycetes, form endospores, the cell being transformed into an ascus (Fig. 79). Schwann, in 1839, first observed spores in yeast. They are not mentioned again until 1868, by de Seynes, and were described for several species by Reess in 1870. The most contradictory views prevailed with regard to the conditions of their formation until Hansen published his researches in 1883. For example, the belief up till then had been that only enfeebled cells washed in water were capable of forming spores, and that the most favourable temperature lay near the freezing point, whereas Hansen showed that this view was entirely erroneous. He found, on the contrary, that spore formation does not, as a rule, take place under these conditions, and that young, well-nourished cells must be sown if a strong spore formation is to be obtained. According to him, further essential conditions are: abundant moisture, plenty of fresh air and a comparatively high temperature (for most species yet investigated, 25° C. is a good temperature). He carried out thorough and comprehensive experiments, particularly on temperature conditions; these will be described later on. His methods of spore culture are described in Section II., p. 121.

There are species of which the young cells form spores under almost all conditions of cultivation, even under such conditions as are very unfavourable for the majority of them. A growth consisting of old cells gives a less vigorous spore formation; if the growth is very old it does not sporulate at all.

Quite recently Hansen made additional researches on this point, and has, among other things, defined the difference between spore formation and budding. The result of these investigations is comprised in the following: If young, vigorous cells are brought into a thin layer of water to which air has free access, colonies are formed (even if all the culture liquid has been removed from the cells) at first by budding; hereafter spore formation takes place, beginning first in the mother cell and extending from this to the younger members of the colony. After several days spores are generally also found in the youngest cells, *i.e.*, such cells as have not put forth buds. It may be seen from this that the yeast cell can produce spores directly without previously forming buds. This also happened when cultivating a wine yeast in a solution of calcium sulphate. But the most remarkable fact brought out by Hansen in this connection is that the spore itself can occur as a spore mother cell. This happens when the spore, after being a short time in a culture liquid containing sugar, has swelled up and is then

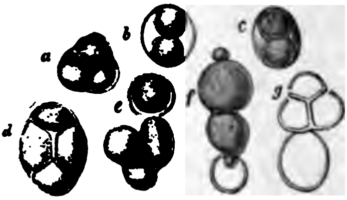


FIG. 80.—*Saccharomyces cerevisiæ I.*, Hansen. Spores at commencement of germination. Formation of a septum may be seen at *a*, *d*, *e* and *g*. In *e*, *f* and *g* the walls of the mother cells have burst; *g* shows a septum formed by the coalescing of three spores into a three-winged spore body; the enclosing wall of the latter is burst in three places. $\frac{1000}{1}$. (After Hansen.)

transferred to an aqueous solution of calcium sulphate. No buds are then produced, and instead spores are formed in the interior of the swollen spore.

Spores, like vegetative cells, consist of a membrane which encloses protoplasm and a cell nucleus. They have the same soft consistency as the latter, but possess greater power to resist drying, heating, etc. Their shape is varied; they are most frequently spherical (*e.g.*, *Sacch. cerevisiæ I.*, Figs. 79 (1), 80, 81, 82 and 89), with greater or less tendency to an ellipsoidal shape; in particular species they are kidney-shaped (*e.g.*, *Sacch. Marxianus*), in others "hat-shaped" (*e.g.*, *Sacch. anomalus*, Figs. 83 and 102), *i.e.*, shaped like the segment of a sphere with a projecting rim round the edge. In some a highly refractive body is found in the middle of the spore (*e.g.*, *Sacch. hyalosporus*). The number of spores in a cell varies from one to eleven (Fig. 79).

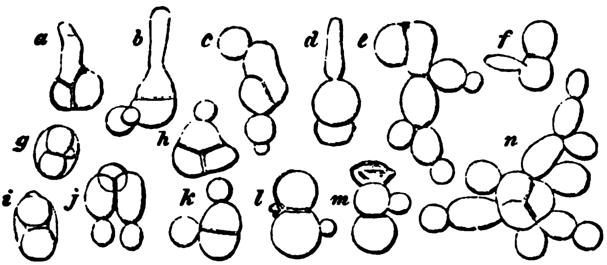


FIG. 81.—*Saccharomyces cerevisiæ I.*, Hansen. Germination of old spores. $\frac{1000}{1}$. (After Hansen.)

Hansen found that there was a difference between culture yeast and wild yeast in the structure of the spore plasma. The spores of culture yeast appear to be empty, while, on the other hand, the spores of wild yeast are strongly refractive. This difference is of importance in the analysis of brewery yeast, although the details have not yet been specified.

An appearance often observed in the spore-bearing cell is the growing together of the spore walls, a septum being thus formed (Fig. 80, *g*). The cell can thus be changed into a many-winged spore body, its walls forming one unit. Sometimes also pseudo-septa are formed between the spores by the latter compressing the plasma which lies between them (Figs. 79, *a*, and 80, *a*, *d*, *e*).

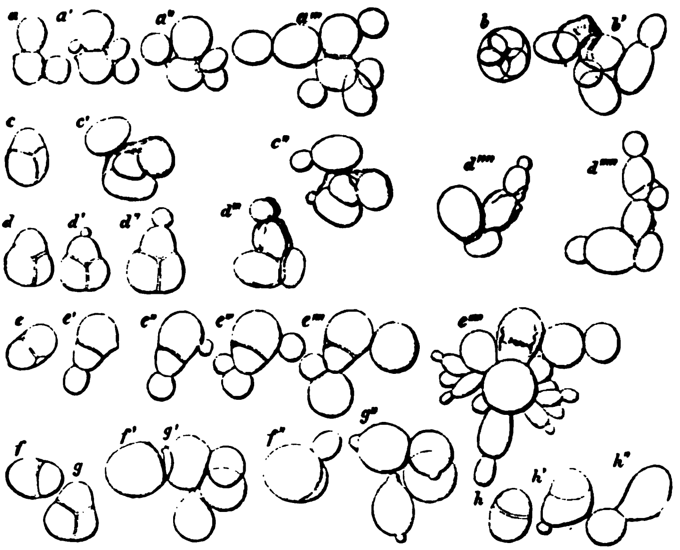


FIG. 82.—*Saccharomyces cerevisiæ I.*, Hansen. Germinating spores. The series *e*-*e'''''* was cultivated on wort gelatine, the others in wort. Temperature about 20° C.; *a* and *b* dried some time beforehand. Time data reckoned from beginning of experiment. *a*, Three spores connected, no mother-cell wall; *a'* after 19 hrs., *a''* after 22, *a'''* after 30. *b*, A cell with four spores; *b'* after 18 hrs. *c*, A cell with four spores; *c'* after 9 hrs., *c''* after 10½. *d*, A cell with three spores; *d'* after 10½ hrs., *d''* after 13, *d'''* after 17, *d''''* after 21, *d'''''* after 25. *e*, A cell with two spores; *e'*-*e'''''* after 7½, 8½, 11, 20 and 50 hrs. respectively. *f* and *g*, Two cells with spores; *f'*, *g'* after 22 hrs., *f''*, *g''* after 25. *h*, A cell with two spores; *h'* after 9 hrs., *h''* after 13; in *h''* the wall between the two spores has disappeared, and both have grown into one. $\frac{1000}{1}$. (After Hansen.)

Spores free themselves from the mother-cell by swelling up and causing the wall of the mother-cell to burst.

Hansen found the following two types of germination:

First type: germination takes place like ordinary budding, and can occur at any point of the surface of the spore (Figs. 80, 81, 82 and 83).

Sometimes it begins while the spore still lies in the mother-cell. Septum formation usually takes place. Sometimes, when the spores grow together, one takes nourishment from the others, and accordingly acts as a parasite. Examples: *Sacch. cerevisiæ I.* (Figs. 80, 81 and 82), *Sacch. Pastorianus I., II.* and *III.*, *Sacch. ellipsoideus I.* and *II.*, *Sacch. anomalus* (Fig. 83).

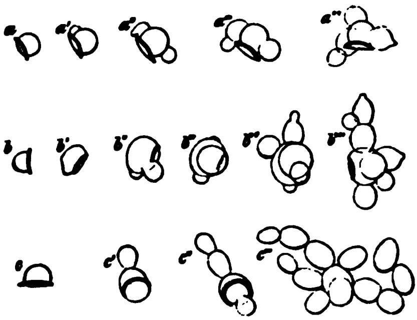


FIG. 83.—*Saccharomyces anomalus*, Hansen. Spores germinating from an old, partially dried gypsum block culture. Cultivation took place in dilute wort: *a* at 28° C., *b* and *c* at 23° C. *a'-a''''* after 7, 12, 15 and 20 hrs. respectively; *b'-b'''''* after 10, 21, 24, 25 and 27 hrs. respectively; *c'* after 8, *c''* after 10, and *c'''* after 21 hrs. $\frac{1000}{1}$. (After Hansen.)

Second type (Figs. 84 and 85): Two or more spores generally grow together in the very first stages of germination, yet old spores are able to germinate individually without growing together. Germination begins with a wart-like or sausage-shaped lengthening which grows on and often occurs as germ threads or bunches. Only from this promycelium is the development of the yeast cells effected later on, a partition wall being first formed between the promycelium and the young yeast cell; the latter is detached by fission of the wall. Example: *Sacch. Ludwigii* (Figs. 84 and 85).

By far the most saccharomycetes belong to the first type.

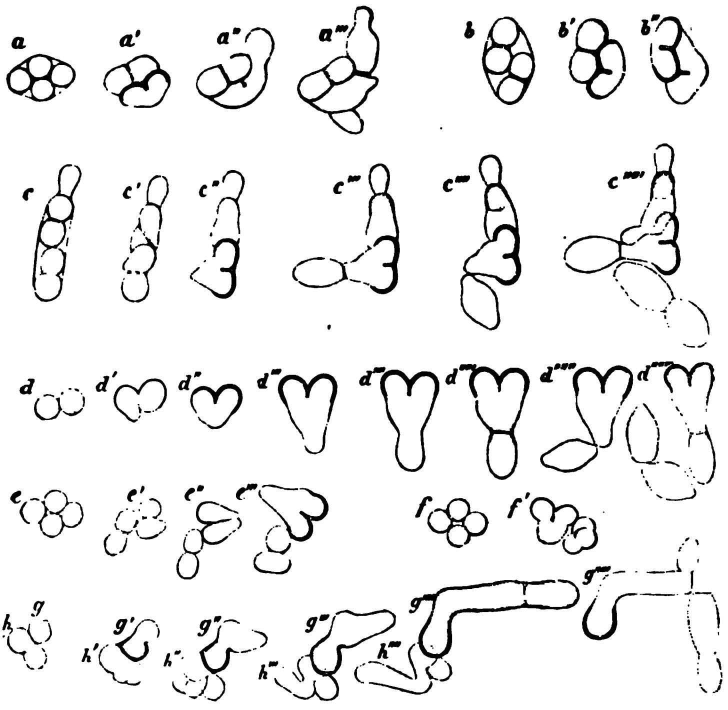


FIG. 84.—*Saccharomyces Ludwigii*, Hansen. Germination of spores from a gypsum block culture: *a*, *b* and *c* were 12 days old at 25° C., *d*, *e*, *f* and *g* were 1½ months old at the room temperature. The cultivation was made in wort, *a* at 25 C., the others at 18° to 20° C. *a*, A cell with four spores; *a'* after 8 hrs., *a''* after 25, *a'''* after 26. *b*, A cell with four spores in two groups; *b'* after 9½ hrs., *b''* after 12. *c*, A cell with four spores in two groups; *c'*-*c'''''* after 12, 15. 20, 24 and 27 hrs. respectively. *d*, Two free spores; *d'*-*d'''''''* after 18, 20, 26, 28, 29, 30½ and 33 hrs. respectively. *f*, Four free spores; *f'* after 19 hrs. *gh*, A group of three spores, of which the two lowermost, *h*, were connected, but are separated from one another by fission: the uppermost spore, *g*, has separated itself in the same way from a fourth spore; *g'h'* after 17 hrs., *g''h''* after 21, *g'''h'''* after 23, *g''''h''''* after 26½, *g'''''* after 28. The lowermost spore in this group did not develop. $\frac{1000}{1}$. (After Hansen.)

Just as Hansen's investigations on film formation have been incorporated in the system of yeast analysis, so also have his above experiments on spore formation been fruitful as regards important characteristics for distinguishing groups and single species; he based on this his method of analysing brewery yeast described in Section II., p. 134. It was found, first, that in different species at the same temperature spores begin to form after different time intervals, and, secondly, that the temperature limits of spore formation are different for different species. Hansen thus determined the spore curves for six species by observing for a series of different temperatures the times at which the formation of spores first began. The cardinal points, *viz.*, the maximum, optimum and minimum temperatures, have special significance, and of these particularly the first and last. The two following examples may serve as illustrations of such spore curves:—

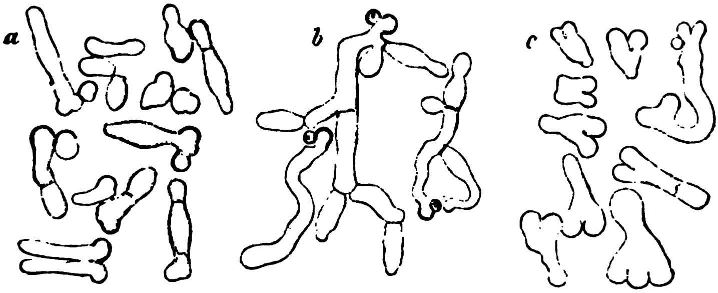


Fig. 85.—*Saccharomyces Ludwigii*, Hansen. Germination of spores from an old gypsum block culture. The germination took place in dilute wort. *a* and *b*, Groups of spores in which each spore has developed its own germ thread. *a* represents the first stages of germination, *b* a further development; in the group *c* various forms of coalescence may be seen. $\frac{500}{1}$ to $\frac{800}{1}$. (After Hansen.)

Sacch. cerevisiae I.

At 37½° C. no spores develop.
„ 36-37° C. first indications appear after 29 hours.
„ 35° C. „ „ „ „ 25 „
„ 33½° C. „ „ „ „ 23 „
„ 30° C. „ „ „ „ 20 „
„ 25° C. „ „ „ „ 23 „
„ 23° C. „ „ „ „ 27 „
„ 17½° C. „ „ „ „ 50 „
„ 16½° C. „ „ „ „ 65 „
„ 11-12° C. „ „ „ „ 10 days.
„ 9° C. no spores develop.

Sacch. Pastorianus I.

At 31½° C. no spores develop.
„ 29½-30½° C. first indications appear after 30 hours.
„ 29° C. „ „ „ „ 27 „
„ 27½° C. „ „ „ „ 24 „
„ 23½° C. „ „ „ „ 26 „
„ 18° C. „ „ „ „ 35 „
„ 15° C. „ „ „ „ 50 „
„ 10° C. „ „ „ „ 89 „
„ 8½° C. „ „ „ „ 5 days.
„ 7° C. „ „ „ „ 7 „
„ 3-4° C. „ „ „ „ 14 „
„ ½° C. no spores develop.

Other investigators have published similar curves for other species. Those given by Will for four species of brewery bottom yeasts are especially noteworthy and will be considered more fully in the systematic description.

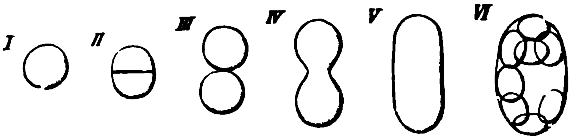


FIG. 86.—*Schizosaccharomyces octosporus*, Beijerinck. Formation of the ascus. I., a round cell shortly before the formation of the septum. II., III., IV., V. and VI., after 1, 3, 6, 10 and 17 hrs. respectively. The times are reckoned from the commencement of the observation. $\frac{1000}{1}$. (After Schiönning.)

It may be seen from the above two series of numbers that spore formation proceeds very slowly at low temperatures, but more quickly as the temperature rises until a certain point, namely the optimum, is reached, after which spore formation proceeds the more slowly the nearer the maximum temperature is approached.

Hansen's experiments on the effect of temperature led him to state the general law that the maximum temperature for the formation of spores in Saccharomycetes always lies several degrees lower than that for bud formation and the minimum temperature a few degrees higher. He determined the temperature limits for budding and spore formation in eleven species. In these species the maximum temperatures for budding lie between 47° and 34° C., the minimum temperatures between 3° and ½° C., the maximum temperatures for spore formation between 37° and 28° C., and the minimum temperatures between 11° and 3° C. Those species which have the highest temperature maxima for budding and spore formation have the same also for film formation.

Schiönning has observed a peculiar mode of ascus formation, in a single species belonging to the genus *Schizosaccharomyces*, *viz.*, *Schizosacch. octosporus.*

This takes place in the following manner: The cell I. (Fig. 86) enlarges in one direction and forms a septum (II.). After a certain time fission takes place. The two new cells now lie either touching one another or connected together at one point (III.). In a further stage both cells gradually coalesce so as to form a cell shaped like an hour glass (IV.). The cell grows and the hour glass shape disappears (V.); finally it assumes an ellipsoidal shape and the ascus is formed (VI.). The spores are then formed in the latter.

4.—*The Chemical Constituents of the Cell.*

The cell membrane consists, according to Casagrandi, probably of pectose or perhaps of an analogous pectin substance, and the chief constituents of the protoplasm consist of albuminoids. Glycogen and fat are frequently present in large quantity.

Errera (1885) first showed that yeast cells contain glycogen. Kayser and Boullanger found that, with a plentiful air supply, there is always less glycogen formed than with a small air supply. The greater the amount of sugar present or the weaker the acidity of the substratum the more glycogen is formed. Large doses of tartaric acid are said to be very effective in preventing formation of glycogen. Otherwise the constituents of yeast cells are substantially *membranæfaciens*. Of the sugars, dextrose, d-mannose and d-galactose are fermented directly, the others only after previous hydrolysis by means of enzymes. E. Fischer has recently, by his chemical researches, made important contributions towards the solution of these difficult questions. Whereas formerly only one enzyme was known to be present in yeast, *viz.*, invertin or invertase, which has the power of breaking up saccharose into dextrose and fructose, and melitriose into melibiose and fructose, Fischer found (1894) another enzyme, *viz.*, yeast glucase or yeast maltase, which turns maltose into dextrose. The reason why this enzyme was not discovered sooner may be explained by the fact that it cannot be extracted from the uninjured yeast cell. According to Fischer this conversion of maltose into dextrose goes on in the interior of the yeast cell. A third enzyme, which is found chiefly among species of bottom yeast, is melibiase, which breaks up melibiose into dextrose and galactose. It follows from Fischer's investigations that hydrolysis precedes all fermentations of polysaccharides, and that the action of yeast on sugar is of a purely chemical nature. He further states the following law: For a yeast to be able to attack a sugar the stereo-chemical structure of the albumen molecule of the yeast cell must not differ to any great extent from that of the sugar molecule.

These discoveries preceded those on the alcohol-forming ferment by E. Buchner (1897), who gave the latter the name zymase. By subjecting the yeast to great pressure he succeeded in obtaining a juice which was able to cause fermentation in sugar solutions.

The enzyme lactase, which breaks up lactose into d-galactose and dextrose, seldom occurs in saccharomycetes. Only those yeasts which contain this enzyme are capable of fermenting lactose. Duclaux (1887) was the first to find a yeast which ferments lactose, and later investigators have since described others. But in most cases it cannot be decided whether these are typical saccharomycetes, as it is not stated whether the species form spores or not. Jörgensen has quite recently described a species of this kind, *Sacch. fragilis.*

Besides the above sugar-transforming ferments, saccharomycetes also contain a ferment similar to trypsin, which can peptonise gelatine, the latter substance being liquefied in the process. This may be observed when yeast cells are seeded on culture gelatine and the culture has attained a certain age. This proteolysis, as it is called, of yeast varies in different species. A study of it has been made by Beijerinck, Wehmer and Will.

One of the most constant characteristics of the saccharomycetes is the enzyme content. A yeast species cannot, as Dubourg states, be brought by culture to such a state as to ferment a sugar which it could not previously ferment. It is also impossible by any treatment to cause the various species to lose those enzymes which they contain. The cell can, however, by means of a certain nutrition, be made to ferment more or less of a particular sugar. The author has carried out experiments on different yeast species, following Dubourg's method of procedure, but always with negative results.

Influence of Air and Temperature on Fermentation.—Among the factors which exert a considerable influence on the progress of fermentation, besides that of the chemical composition of the liquid, those of temperature and the amount of oxygen present in the liquid may be mentioned. The usual limits of temperature within which fermentation may take place at all are 0° and 40° C. This much is known of the effect of oxygen on the progress of brewing in practice—that, in order to get a good result, it is a necessity to aërate the wort, *i.e.*, to let it take up oxygen from the air. Pasteur's researches on this subject led him to state the theory that fermentation is life without air and that yeast can only decompose sugar by taking the necessary oxygen from the sugar molecule. His theory was, however, proved to be wrong, as we have seen. He found that by supplying oxygen multiplication is favoured, but fermentation is restrained, and further that wort takes up oxygen from the air in two ways, *viz.*, partly by forming a mechanical mixture with it and partly by entering into chemical combination with it. A deeper insight into the influence of aëration on fermentation in breweries could only be obtained by the study of pure culture species and races, for, as Hansen's investigations in brewery practice have shown, they behave differently with respect to oxygen (see, for instance, his experiments on Carlsberg bottom yeast Nos. 1 and 2). Korff recently obtained similar results. He experimented on the three yeast races Saaz, Frohberg and Logos, cultivating them in a 10 per cent. solution of saccharose with an addition of yeast water or Hayduck's asparagine solution. By passing in air, oxygen and hydrogen respectively, the three species then showed very different behaviours as regards their energy of fermentation, fermenting power, energy of multiplication and power of multiplication.

In this respect there are three things which must be considered, *viz.*, the chemical condition of the wort, the effect of aëration on the yeast species in question, and the demands which the finished product must satisfy. Every brewery must therefore find out by trial that method of aërating the wort which gives the best result under the prevailing circumstances. There are no definite general rules for this. Hansen mentions a remarkable experiment, which showed that wort can be in such a condition that it need not be subjected to the usual aëration in the brewery, which was formerly considered quite indispensable to obtain a good fermentation and a clear beer. But such a chemical condition of the wort is very exceptional. The experiment was made on Carlsberg bottom yeast Nos. 1 and 2 in ordinary lager beer wort. The variation thus caused in the yeast cells will be treated later.

In course of time many investigations, practical and theoretical, have been made into this behaviour. As an example of the former, the experiments of Anton Petersen in the old Carlsberg brewery on Carlsberg bottom yeast No. 1 may be cited, the result of which was that those brews which contained a large amount of oxygen showed on an average a greater attenuation after the primary fermentation than those which contained less oxygen.

The numerous theoretical investigations which have been undertaken since Pasteur (*e.g.*, by Nägeli, Rasmus Pedersen, Hansen and others) on the effect of aëration on the yeast cell and on fermentation, show that aëration exercises a favourable influence on the total energy of the yeast in consequence of the increase of the multiplying power, but that, under these circumstances, the individual cell forms less alcohol than when no aëration takes place. Hansen found further, that when the yeast cells have free access to the oxygen of the air, or even when surrounded by it, they can yet produce an active fermentation, an observation which contradicts the theory of Pasteur mentioned above. The latter also holds for the experiments of Adr. J. Brown, Giltay and Aberson. Brown observed that a plentiful supply of oxygen increases the fermentative activity of the single cell, even when the cells are in such circumstances as prevent multiplication.

Excess of oxygen slows the fermentation if the yeast has already reached the maximum of its multiplication, or if the nutrient solution was charged from the beginning with a quantity of yeast exceeding the maximum amount (Prior).

Finally, it may be added that when aëration is so strong as to cause a violent commotion, its influence then becomes disadvantageous, and very markedly so with defective nutrimental conditions and yeast species of small fermenting power (Buchner and Rapp).

Energy of Fermentation and Fermenting Power.—By activity or energy of fermentation is understood the intensity with which a yeast can decompose a sugar within a certain time. It of course varies in the different yeast species. Prior has determined it for some species by Meissl's method; the latter consists in noting the weight of carbonic acid which is liberated by 1 gram of yeast from a sugar solution of a certain composition[1] in six hours at 30° C. According to Meissl, a normal yeast is one which liberates 1·75 gram of carbonic acid gas under the above conditions; the energy of fermentation of this is then put down as 100. Prior found the following values for the given species:—

Carlsberg bottom yeast No. 1	136·40
„ „ „ No. 2	106·13
Sacch. Pastorianus I.	155·48
„ „ *II.*	280·72
„ „ *III.*	202·20
„ *ellipsoideus I.*	285·76
„ „ *II.*	219·03

Permeability of the Cell Membrane.—Since the transformation of the sugar into fermentable sugar takes place in the interior of the cells, the energy of fermentation is thus also a measure of the permeability of the cell wall (Prior). This varies according to the age and condition of the cells, and depends, besides, on the power of the latter to form fungous mucilage, since the permeability diminishes the more this substance is given off. The permeability varies, of course, with the different kinds of sugar. For instance, Prior obtained the following result for Carlsberg bottom yeast No. 2:—

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[1] 4·5 grams of a mixture of 400 grams of candy sugar, 25 grams of ammonium phosphate and 25 grams of potassium phosphate are dissolved in 50 c.c. of tap water.

Saccharose.	Dextrose.	Fructose.	Maltose.
106·13	87·09	73·67	69·71

It may be seen from this that saccharose had the greatest diffusing power.

Products of Fermentation.—Besides ethyl alcohol and carbonic acid gas, the saccharomycetes form, during fermentation, other substances also, although in smaller amounts, *viz.*, glycerine and succinic acid. Zopf discovered a species, *Sacch. Hansenii,* which forms oxalic acid. Raymann and Kruis have proved that, under certain conditions, the culture yeasts employed in the manufacture of spirits form amyl alcohol. In addition to these, volatile organic acids are formed, *e.g.*, acetic acid and volatile, ester-like bouquet substances. The quantity of these substances varies according to the conditions under which the fermentation takes place, and their quality also varies in the different species and races, so that the product formed by the latter is extremely variable. Having regard to this, the necessity urged by Hansen for the systematic selection of races in practice will be recognised. According to Prior, the wild yeast species (*Sacch. Pastorianus I., II.* and *III., Sacch. ellipsoideus I.* and *II.*) form larger amounts of volatile than of fixed acids, whereas with culture yeasts the opposite is usually the case. The ester-like substances produce a strong taste and smell in the finished product, even when present in small quantities, these substances, which the various species produce, being widely different from one another. Thus *Sacch. anomalus* brings out a strong taste a smell of fruit ester, while some of the disease yeasts discovered by Hansen develop a very strong bitter taste and disagreeable smell.

There are formed in wine, by the different wine yeasts, those volatile compounds which Wortmann calls secondary bouquet substances (fermentation bouquet), and which are the substantial cause of the special taste of wines from particular places. These by-products are therefore of no little importance.

Many yeasts are capable of a reducing action, forming sulphuretted hydrogen when sulphur is present during fermentation. Other species are able to form sulphurous acid in must (B. Haas, W. Seifert) and also in wort (Schwackhöfer, Will). Many wine yeasts have an acid-consuming action (Schukow, Wortmann), gradually using up the organic acids present in the wines. (*Cf.* W. Seifert's researches mentioned later on.)

Auto-Fermentation.—When thick liquid yeast is set aside at a favourable temperature, it may be seen that, although no culture liquid is present, alcohol and carbonic acid gas are formed. This phenomenon is called auto-fermentation. It takes place through the yeast transforming its self-contained food stuffs. According to Lintner, the glycogen in the yeast cell is, in auto-fermentation, apparently turned first into sugar, and this then fermented into carbonic acid and alcohol. During auto-fermentation yeast gives off a smell, more or less strong, of fruit ether, which probably arises from esters of the higher alcohols.

Yeast Types.—In fermentation industries various yeast species occur which exhibit different actions during fermentation. We shall now consider yeast types from this point of view. The intensity with which the sugars are attacked by the yeast species varies, as we have seen. At the very beginning, when Hansen introduced his pure culture system into the brewery, he proved that there was a difference in this respect between the two brewery bottom yeasts named by him Carlsberg bottom yeast No. 1 and No. 2. Several such types were found later; the Berlin station has established the following three, *viz.*, Saaz, Frohberg and Logos. These are thus characterised by Prior: The Saaz yeasts in a fermentation leave unfermented most achroodextrin III., and consequently also more maltose than those of the Frohberg type, which again leave more than the Logos yeast. Prior, however, does not recognise the Saaz and Frohberg types in the physiological sense of fermentation. According to this author the same degree of fermentation is finally reached with both, if the fermentation is conducted under the most favourable conditions (large yeast supply, high temperature, strong aëration).

Top fermentation is one in which the froth on the surface is often covered with a thick layer of yeast; in bottom fermentation this layer is never thick, and is sometimes entirely absent. In typical top and bottom fermentations this, the really only noticeable point of difference, is very prominent. Various investigators have attempted to find definite pronounced characteristics for each of these groups; but just as there are species which, with respect to the phenomenon of fermentation, stand between both categories, so is it also with those properties which have been classified as special characteristics. A. Bau considered he had found a distinctive property of the bottom yeasts in the fact that they completely ferment melitriose (raffinose), whereas top yeasts are unable to do this. The test applies generally, so far as his own experiments go, to forms included under *Sacch. cerevisiæ;* but recent experiments by himself and by Schukow have shown that most of the typical bottom yeast forms among the wine yeasts cannot completely ferment melitriose, and the above characteristic has thus undergone a considerable limitation. The action is therefore as follows: The enzyme invertase present in yeast decomposes the melitriose into melibiose and fructose; the top yeasts especially are only capable of fermenting the latter of these (Bau). On the other hand, most bottom yeasts contain the enzyme melibiase which breaks up melibiose into dextrose and galactose, both of which are fermented by most bottom yeasts (E. Fischer).

Sometimes a bottom yeast may for a time exhibit feeble signs of top fermentation (Hansen, Kühle). In this respect, therefore, no absolute boundary can be drawn between top and bottom yeast. It is certain, however, that no one has hitherto been able to transform a typical top yeast into a permanent typical bottom yeast, and *vice versâ*. It used to be a general belief that by the cultivation of a top yeast at a low temperature it could be transformed into a bottom yeast. But Hansen has cultivated such typical top yeasts as *Sacch. cerevisiæ I.* and *Sacch. Pastorianus III.* for several years at a temperature of 5° to 7° C. without anything happening except that, as one might expect, the fermentation was feebler; but as soon as the cultivation was continued again at a high temperature, the signs of top fermentation became as prominent as before. Conversely, Hansen has cultivated typical bottom yeasts such as *Sacch. Pastorianus I.*, *Sacch. ellipsoideus I.* and *II.*, Carlsberg bottom yeast No. 1 and No. 2, and several others for years at ordinary room temperature, *i.e.*, at a temperature considerably higher than that usually employed in bottom fermentation breweries, without any signs of top fermentation ever appearing. In earlier times the view was held that if a bottom yeast was allowed to form a film, it was then turned into a top yeast. Hansen has shown, by exact experiments, that this also is entirely false.

Culture yeasts is the name given to such yeast species as have for long been cultivated in fermentation industries, though only recently in a systematic manner and under a certain amount of control. As the consumption of beer is now extremely widespread, culture yeasts will, of course, be met with in nature; most yeast species, however, which occur either on fruits or in the earth belong to so-called wild yeast species. Culture yeasts are, like wild yeasts, partly top yeasts and partly bottom yeasts. Their use depends on certain special qualities for which they are prized, *e.g.*, in breweries, when they give a stable beer, a good clarification, a particular flavour, etc. A yeast which gives a stable beer must not only multiply comparatively slowly in the finished beer, but must also be able to suppress rival yeasts during fermentation. The immediate cause why certain species are more pre-eminent in the latter respect may, in some cases, be sought in the fact that they can fit themselves to nutrimental conditions, and especially can assimilate oxygen more powerfully than their rivals; in other cases it is due chiefly to them giving off substances during multiplication which act as poisons on the intruders.

Since the demands made regarding taste and other qualities vary so widely the choice of available species and races must necessarily adjust itself accordingly, whether it be for wine manufacture, or for breweries, distilleries or pressed yeast factories; thus the number of species and races introduced into practice continually increases. In the first two branches of industry the demands are more for clarification, taste, smell and stability: in the latter a large production of alcohol and great multiplying power are more particularly sought.

6.—*Injurious and Stimulating Influence of Chemical and Physical Factors.*

Influence of Chemical Factors.—With saccharomycetes, as with all other organisms, the decomposition products act as poison to the organism in question. Here it is the alcohol formed, and the organic acids, among other things, which exercise an injurious influence on their growth. The deleterious effect of carbonic acid, however, does not appear to be great.

As regards the action of organic acids, the experiments of Hansen on the effect of tartaric acid have already been mentioned (see p. 135). He found that beer yeasts die off very quickly if they are cultivated in a sugar solution to which tartaric acid has been added, an effect not produced on wild yeasts.

The stimulating effect of antiseptics on yeast has been studied by earlier investigators, *e.g.*, by Biernacki and Schulz (antiseptics in general), by Hayduck (sulphuric and lactic acids) and by Heinzelman (salicylic acid). Agents were thus found, by means of which not only could the development of bacteria be prevented, but the energy of fermentation also increased. Now and then experiments in practice were also instituted. In this direction Effront has quite recently recommended the use of fluorides. Hydrofluoric acid is, however, a strong poison for yeast which can hardly withstand 1 to 2 grams per hectolitre; but, by adaptation, the dose can be raised to 200 grams. A yeast habituated to such large quantities of hydrofluoric acid possesses indeed but feeble powers of budding, although it has great fermenting power. The method is only applicable in distilleries. According to Holm and Jörgensen's experiments, this addition of fluorides hastens the development of *Mycoderma*, and, at the same time, *Bacterium aceti* is not thereby destroyed. In mixtures of brewery yeast with wild yeast the same authors state that the development of the wild yeast is favoured, the effect being thus the same as that of tartaric acid. This method also is, therefore, quite inapplicable to the "purification" of beer yeast.

Sulphurous acid, corrosive sublimate and several other substances are likewise strong yeast poisons. Thus alcoholic fermentation is, according to Yabe, prevented by the following solutions: Phenol 1 : 200, resorcin 1 : 100, pyrogallol 1 : 50. Bokorny gives: sulphuric acid 1 : 5,000, potassium hydroxide 1 : 5,000, potassium permanganate 1 : 10,000, chlorine 1 : 10,000, and iodine 1 : 10,000. Siebel found that if beer is treated with a solution of formalin (40 per cent. solution of formaldehyde) in the proportion 1 : 10,000, neither yeast, *Mycoderma* nor bacteria develop; in solutions of 1 : 50,000, yeast and *Mycoderma* develop but not bacteria.

Influence of Physical Factors.—As regards the influence of physical causes, yeast cells do not usually withstand 50° to 60° C. moist heat, but die off between these two temperatures. Hansen found that strong young cells of *Sacch. ellipsoideus II.* die after five minutes' heating in distilled water at a temperature between 54° and 56° C. Old cells of the same species could withstand a temperature of 60° C. for five minutes under the same conditions without being killed. Quite ripe spores of the same species, which had been partially dried on a gypsum block for about a week, withstood five minutes heating in water at 62° but not at 66° C. Similar experiments were made with *Sacch. cerevisiæ I.*: strong young cells could withstand five minutes heating at 52° C. under the same conditions, but not at 54° C., and spores treated in the same manner as those of the foregoing species could withstand five minutes heating at 58°, but not at 62° C. Yeast cells are said to be able to stand cooling down to − 130° C. for about 200 hours, and it is known from experiments in breweries that yeast cells can remain frozen in ice for months at a time without being killed (Prior).

Drying and the effect of temperature are related to one another. Experiments by Hansen and others have shown that yeast cells die comparatively quickly when dried up, and that this occurs in nature, *e.g.*, when the cells are on the uninjured surface of fruit. Under these circumstances the spores have a somewhat longer life than the vegetative cells, but the difference is not great. By a suitable treatment, however, life can be preserved in the dried cell for a long time. This will be mentioned under the heading following.

The injurious effect of violent shaking on yeast cells has already been touched upon (p. 217).

Experiments on the effect of light have been made from time to time by various investigators. Kny used as the source of light five flat gas flames, and carried out his experiments on pressed yeast (*Sacch. cerevisiæ*) which was placed in an artificial culture solution in flat crystallising dishes and at the same temperature, part exposed to the action of light, and part set away in the dark. The heat from the source of light was removed by means of water. He arrived at the result that the budding of *Sacch. cerevisiæ* takes place in this moderate light with the same activity as in darkness. Marshall Ward remarked a destructive effect of light on the spores of *Sacch. pyriformis*. Lohmann made experiments with intense light (arc lamps and sunlight) and excluded the heat radiated from the sources of light by means of water cells. A distillery yeast, called Race II., was seeded in wort gelatine on glass slips and for eight hours at constant temperature part was subjected to light from the arc lamp, and another part was set away in the dark. For high temperatures (*i.e.*, above 18°) it was found that budding was retarded in the cultures subjected to the light. The same yeast was also seeded on agar-agar plates in Petri dishes, a part exposed to sunlight and another part kept in darkness. Illumination for several hours in this case killed the yeast. Diffused daylight also had a retarding influence on budding, but only after prolonged action. It resulted from experiments with *Sacch. Pastorianus I.* that this species has greater resisting power than the above one against the effect of light, both from the sun and from the arc lamp.

It has long been known in the brewing world that beer is sensitive to light in a high degree. Special experiments on this point were made by Ney, Beck and W. Schultze. The question put before one here is whether the disagreeable smell and taste, which beer gets when exposed to sunlight, originates in the effect of light on the yeast or on the beer itself. At the time when the first two named carried out their experiments (1878 and 1882), *Sacch. exiguus* was always looked upon as the cause of everything that went wrong, and it was found, in concordance with the above, that in those flasks least protected from light, and the contents of which had therefore acquired a very bad smell and taste, there was a more or less copious development of "abnormal yeast cells," which they set down as the above-named species without further ado, and also of lactic acid bacteria. From this it would seem then that light was the cause of the good beer yeast, with which the beer was treated, being restrained and the supposed *Sacch. exiguus* being furthered in its development. But from the communications it appears rather to have been *Sacch. Pastorianus I.* or an allied species, and since the latter shows a greater power of resistance to the effect of light than *Sacch. cerevisiæ*, as we have seen from the above experiments of Lohmann, it is not impossible that this species was responsible for the disagreeable smell and taste in the cases cited, since the action of the light in the experiments was very prolonged (three weeks). But it is not yet clear what the *rôle* played by the yeast cells in these undesirable transformations really is.

—*Vitality of Yeast in Nutrient Solutions and in the Dry State.*

Hansen's researches have likewise furnished explanations of the vitality of saccharomycetes in nutrient solutions. The results were the following: The best preserving liquid for these fungi is, as stated in Section II., a 10 per cent. solution of saccharose. In the course of more than twenty years' observations, and in experiments with forty-four species and varieties, the only ones which died in this solution were *Sacch. Ludwigii*, Carlsberg bottom yeast No. 2, and its asporogenous variety, in several cases, however, only after some years, and, indeed, only some of the growths; as regards all the other species and varieties no dying off was observed, although the majority of them had been sixteen to seventeen years and several of them more than twenty years in the sugar solution. The behaviour of saccharomycetes is essentially different in wort, and here great irregularities prevail. In one case the same species died frequently in five months, in another it still lived after twelve years; usually death occurred early. In preservation in water the extent of the seeding is of great moment, *i.e.*, whether it is a small or a large one; in the first case the species were dead after one and a half to two years, in the latter they were still living after ten years. The stronger cells, in fact, live at the cost of the weaker ones. The resisting power of yeast towards drying varies very much also; this depends mainly on whether the single cell is subjected to the drying or the cells lie together in large quantity and thus form a thick layer. Hansen drew attention to this in 1885. Later on he made experiments on the resistance of the isolated cell to drying by dipping a piece of platinum wire into a yeast mass, placing the wire in an empty Freudenreich flask and shaking the latter in such a way that the small quantity of yeast on the wire was spread over the bottom and walls of the flask. He thus obtained as thin a layer of yeast as possible. It then resulted that under these circumstances the saccharomycetes remain alive only for a short time. Some species died in less than five days. *Sacch. Marxianus* held out longest, being alive for three months, also *Sacch. anomalus* and *Sacch. membranæfaciens*, which lived eighty and sixty-five days respectively. These facts refer to the vegetative cells. Under the same circumstances spores lived at least five months.

In addition, Hansen made numerous experiments relating to the drying of yeast cells in other ways, *viz.*, when placed in layers on filter paper and when put on cotton wool in small flasks with a wadding plug (Section II., pp. 62 and 117). In the first case, as a rule, the vegetative cells died in the course of a year; under the same circumstances the spores lived for one to two years longer. On the cotton wool, the species tested lived for more than a year, some even more than three years; under these conditions they formed spores, and it is probable that this had something to do with their longer period of life. Experiments in practice on the preservation of brewery yeast by drying have been described in Section II., p. 118.

8.—*Disease Yeasts and Mixed Fermentations.*

Diseases caused by Fermentation Products.—We have seen from the above that yeasts occur which are the cause of diseases in beer. As an example of such a yeast, *Sacch. Pastorianus I.* may be mentioned, which, according to Hansen's experiments (1882), produces a bitter taste and disagreeable smell in bottom fermentation beer. If the quantity of this yeast amounts to $\frac{1}{5}$ that of the stock yeast, the disease is very noticeable; even when it only amounts to $\frac{1}{22}$ the disease is still appreciable. It may, at the same time, have an undesirable action on the clarification, and cause turbidity. Will has isolated two similar disease yeasts in Bavarian bottom fermentation breweries; the one gives the beer a sharp, bitter after-taste and produces strong turbidity, the other causes a somewhat sweet disagreeable aromatic taste, and a bitter astringent after-taste, as well as turbidity.

Diseases caused by Turbidity.—Several wild yeast species produce in beer the disease called yeast turbidity by multiplying rapidly during storage, particularly in bottles, the beer becoming filled with cells, the specific gravity of which is such that they remain suspended. This was demonstrated by Hansen in his experiments with *Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.* (1883). The same holds for these disease yeasts as for the foregoing, namely, that they must be present at the beginning of the primary fermentation in order to be able to cause the disease. An infection, therefore, which takes place only at the end of the primary fermentation, while bringing the beer into the storage cellar, is without effect. If it here amounts to $\frac{1}{41}$ of the latter and the beer is also casked with an extract of 7·5 per cent. Balling, the storage being interrupted after two and a third months, the disease will make its appearance. However, this will not happen if the quantity of extract is reduced to 6·7 per cent. Balling, and the beer is stored for at least three months.

Wortmann has recently drawn attention to the fact that turbidity of a particular kind may make its appearance in wine, by the cell wall of dead cells dissolving, and the contents of the latter being distributed.

Diseases similar to the above have also been observed in top fermentation breweries (de Bavay, Frew).

Competitive Relations.—When two or more species are present together in a nutrient solution they have, as a rule, an injurious action on one another as regards multiplication, a state of competition arising among them.

Hansen was the first to make experiments in this direction. In his paper of 1881 he describes the competitive relations in beer wort between *Sacch. apiculatus* and brewery bottom yeasts. It resulted that when the same number of cells of both species was seeded in the same flask, the multiplication of both was smaller than in the corresponding flasks in which the same number of cells of each species had been seeded separately. The increase of *Sacch. apiculatus* was considerably lessened, but the amount of alcohol formed was the same both in the flask with the mixed seeding, and in that which contained *Sacch. cerevisiæ* only. In another series of experiments, carried out in a similar manner, but with the difference that the seeding of *Sacch. apiculatus* contained twice the number of cells in that of *Sacch. cerevisiæ*, the result was the same as regards the mutual retarding action which the two species exercised on multiplication, but here somewhat less alcohol was formed in the flask with the two species than in that containing *Sacch. cerevisiæ* alone. The increase of *Sacch. cerevisiæ* was also restrained to a greater extent than in the first series of experiments. The experiments were carried out at various temperatures, and with different species of brewery bottom yeast. The main result was that *Sacch. apiculatus*, as the weaker probably at the close of the primary fermentation, is checked in the struggle with *Sacch. cerevisiæ*, but that it can also exercise a restraining action on the increase of its stronger rival and on the alcohol production of the latter. When each species was in a separate flask *Sacch. apiculatus* increased more rapidly than *Sacch. cerevisiæ*; with equally numerous seedings the proportion was 3 : 1.

A very remarkable result was obtained by Hansen in his experiments in practice with mixed fermentations of brewery yeast species, *viz.*, Carlsberg bottom yeasts No. 1 and No. 2. The chief result was that the pitching yeast gives a beer of less stability when it consists of a mixture of two brewery yeast species than when it consists of one of the species only. In these mixtures the species present in smallest proportion acted as a disease yeast, making the beer less stable. The experiments showed that this happened not only when the two species were mixed in the proportion 9 : 1, but even when the proportion was 19 : 1. The disease then appeared only when the storage of the beer was interrupted after 1¼ to 1⅔ month; after three months' storage only a faint indication of it was noticeable.

In his experiments on mixed seeding, Vuylsteke found that when a mixture of *Sacch. cerevisiæ I.* and *Sacch. Pastorianus I.* was seeded in wort, the number of cells of the former species per unit of volume increased from the first to the second day from 1 to 4·81 and 5·18 respectively; the number of cells of *Sacch. Pastorianus I.* rose from 1 to 13·3 and 12·2 respectively. From this the increase of *Sacch. Pastorianus I.* had been $\frac{13\cdot3}{4\cdot81} = 2\cdot76$ and $\frac{12\cdot2}{5\cdot18} = 2\cdot35$ times greater than that of *Sacch. cerevisiæ I.* When a mixture of *Sacch. cerevisiæ I.* and *Sacch. Pastorianus III.* was taken for pitching, the cells of *Sacch. cerevisiæ I.* increased in the proportion 1 to 5·02 and 1 to 4·62 in the first twenty-four hours, and the cells of *Sacch. Pastorianus III.* in the proportion 1 to 3·57 and 1 to 3·01. The increase of the cells of *Sacch. Pastorianus III.* compared with the cell increase of *Sacch. cerevisiæ I.* was thus $\frac{3\cdot57}{5\cdot02} = 0\cdot71$ and $\frac{3\cdot02}{4\cdot62} = 0\cdot65$.

G. Syrée, in a research on the competitive struggle between the culture yeast Frohberg and *Sacch. Pastorianus III.*, has recently obtained the following results: He employed, as culture substratum in his experiments, yeast water with 10 per cent. of saccharose added. At 25° C. the Frohberg yeast when by itself exhibited an energy of multiplication of 1110 (after four days) and a multiplying power of 1659 (after four weeks); at a temperature of 5° to 6° C. the numbers were respectively 383 and 474.

As regards *Sacch. Pastorianus III.* he found, with the same conditions, the following numbers: 921, 1310, 824 and 843. When the two yeast species were together the behaviour was as shown in the following table:—

Proportion of cells in the mixed yeasts.		25° Energy of multiplication.	25° Multiplying power.	5°—6° Energy of multiplication.	5°—6° Multiplying power.
Frohberg.	*Sacch. Past. III.*				
$\frac{199}{200}$	$\frac{1}{200}$	545	767	355	492
$\frac{19}{20}$	$\frac{1}{20}$	705	939	525	656
$\frac{9}{10}$	$\frac{1}{10}$	763	1563	414	787
$\frac{3}{4}$	$\frac{1}{4}$	1116	1560	314	910
$\frac{1}{2}$	$\frac{1}{2}$	1035	1373	866	933
$\frac{1}{4}$	$\frac{3}{4}$	1041	1367	320	912

Wherever the pure culture system has been well and completely introduced into brewery working nothing is to be found which can be called competition between the culture yeast and the wild yeast. The latter cannot make its presence felt and can cause no injury. But even in a brewery where the new system is carried out properly there is a chance of infection by wild yeast from outside, *e.g.*, from the open cooling vessels, from uncleaned mains, etc.; in short, the danger is always present. Therefore, the brewer must, as a matter of course, use precautions to maintain the selected pure culture introduced. Delbrück has recently called this mode of working "the natural pure yeast culture". There are certain old brewery practices to which he has given prominence; according to him it is worth while to use every means to preserve the pure culture and to overcome any infection present, and his remarks on this topic are noteworthy. His experiments led him to the opinion that it may be convenient to make the temperatures of fermentation in the bottom fermentation brewery somewhat higher than formerly. Hansen's researches on the behaviour of yeast at different temperatures form an important basis to Delbrück's natural pure culture. He has shown, namely, as may be remembered, that great differences exist in this relation among the species and races. Immediately after the introduction of the pure culture system, Hansen also communicated the scientific principles of a method for improving stock yeast, which had long been known in breweries. This method consists in taking the fermenting wort at the commencement of the primary fermentation instead of sedimentary yeast during a series of fermentations. The reason why a really better yeast can be thus obtained lies in the fact that, according to Hansen's experiments, the culture yeast preponderates at the beginning of the primary fermentation and the wild yeast at the end.

In this matter there are two things between which proper distinction must be made, the preparation of the pure culture and the maintenance of the latter in the brewery. The natural pure culture has its use and value only in the last named direction; it must not be forgotten, however, that its sphere of action still lies for the greater part in darkness.

9.—*Variation.*

Saccharomycetes are, like all other organisms, subject to variation. The greater part of our knowledge of this subject is due to Hansen, and, in the following, a *résumé* of his investigations on this point is given.

The changes caused by variation are either temporary or permanent; between both extremes intermediate forms are met with.

Temporary Varieties.—On the introduction of the pure culture system the researches in connection with it in the laboratory and in practice gave many opportunities for the observation of different variation phenomena. Such observations are mentioned in several places in Hansen's papers. If a yeast is taken from the brewery and cultivated for some time under laboratory conditions, it easily assumes qualities (greater attenuation, impaired clarification, unusual taste) other than those it formerly possessed. Such phenomena were frequent in former times as, in preparing pure yeast in the laboratory for use in breweries, the conditions in practice were not always sufficiently well imitated. Also in more difficult cases the same wort was not used as in the brewery for which the yeast was prepared. But the new properties acquired in this manner soon disappear again under normal working conditions.

Of other appearances of temporary variation, the following may be mentioned: Hansen sometimes found in a culture of Carlsberg bottom yeast No. 1 on gelatine, colonies which consisted of normal egg-shaped cells, sometimes colonies which contained sausage-shaped cells. Each colony, cultivated by itself, gave a progeny which retained its characteristic cell form. It was only after repeated cultures, finally in a fermenting vat in the brewery, that the sausage-shaped cells disappeared, and the yeast again contained only normal egg-shaped cells. Hansen further found by cultivating *Sacch. Pastorianus II.* on wort gelatine at 25° that vegetations form partly with the appearance of *Sacch. ellipsoideus*, and partly with that of *Sacch. Pastorianus.*

In general, vegetative cells and spores vary very much as regards size and shape, the latter also with regard to the number in the mother cell. Feeble vegetative cells and spores produce small cells. Other peculiar changes of shape have been already mentioned in the description of the growths of film formation.

Hansen's experiments made in 1883-84 with Carlsberg bottom yeast Nos. 1 and 2 have shown that a previous culture in unaërated wort may have an important influence on the clearing power of a brewery yeast in practice, and thereby cause a precursory variation. The first species in particular, after it had passed through repeated fermentations in unaërated wort in the brewery, gave a very poor clarification and an extraordinarily strong fermentation. Under the same conditions yeast No. 2 also gave poor clarification, but returned tolerably quickly to its normal condition, *viz.*, after it had passed through two primary fermentations in the fermenting vats of the brewery. The influence of chemical substances makes itself felt here. This is expressed more plainly by the following example: Hansen observed that the disease yeast *Sacch. Pastorianus I.*, which produces a disagreeable taste and smell in wort, loses this power if kept for a time in a saccharose solution. Another example of a precursory variation, which, however, continued through a few generations, was communicated by Hansen in 1886, when he showed that the film cells of certain species and the cells of old growths which had been developed in a saccharose solution could form, in wort cultures, a loose-lying and often cheese-like yeast sediment quite different to the normal dough-like sediment. This change only disappeared after repeated cultures in wort. Cheese-like yeast can also be formed after prolonged drying.

In his first paper on spore formation (1883) Hansen drew attention to the fluctuations which may appear in this function; these depend partly on the fact that individuals of the same species behave differently, and partly on external factors. For example the cells of *Sacch. Pastorianus I.*, produced in wort at 27° C. in two days, form spores with more difficulty than those produced at the same temperature in one day; the difference is still greater if the cultures are one and seven days old respectively. About six years later Hansen published the discovery of asporogenesis. He had noted that *Sacch. Ludwigii* when allowed to stand in culture media could form cells which had lost the power of developing spores. He further observed that under these conditions the isolated cells developed three vegetative forms: the first possessed the power of vigorous spore formation, the second had almost lost this power, and the third formed no spores at all. The observed deviation from normal behaviour was found to be hereditary in wort cultures for a long time.[1] But by cultivation of the latter two forms in a culture liquid containing dextrose, these quickly resumed their normal condition. The same behaviour was noted afterwards by the author as regards *Sacch. Marxianus.* It appeared later that all asporogenous varieties of *Sacch. Ludwigii* are not affected by dextrose in this way. The above variation of *Sacch. Ludwigii* forms an example of transition from an entirely temporary variation to a permanent one. The effect of dextrose in this case offers, on the other hand, an instance of the specific action of a single chemical substance.

Permanent Varieties.—Hansen prepared in the same year a permanent asporogenous variety showing that *Sacch. Pastorianus I.* completely loses its power of forming spores on being cultivated for several generations in aërated wort at a temperature which is higher than the temperature maximum for spore formation, and only a little lower than the temperature maximum for bud formation. His later experiments have shown that this law holds for all typical saccharomycetes (culture and wild yeasts). The peculiar species *Sacch. membranaefaciens*, *Sacch. anomalus* and *Sacch.*

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[1] Hansen also observed the rise of asporogenous forms in other species when allowed to remain for a long time on culture gelatine and in wort. Beijerinck and P. Lindner have recently made similar observations. Thus Beijerinck has separated two forms of *Schizosacch. octosporus*, an asporogenous and a sporogenous. With the first he found that the formation of trypsin had been very much restricted, and that the formation of acid was greater than in the sporogenous form. Alfred Jörgensen states that top yeasts preserved on gelatine gave a slower clearing and a greater attenuation than under normal conditions.

Ludwigii appear to be the only exceptions; but these species also deviate so very much from true saccharomycetes that they ought probably to be set up as types of new genera.

Quite recently Hansen has published detailed descriptions of his investigations on the formation of permanent asporogenous saccharomycetes by the above mode of culture. The starting point of his experiments was always from a single cell, in some cases a vegetative cell, in others a spore. To find out the conditions of spore formation at various stages during treatment he employed surface plate cultures on wort gelatine (see p. 106). The grown up colonies were then, as soon as their size permitted, placed directly on moist gypsum blocks for sporulation. Those colonies which were too small for this mode of treatment were put in wort and the sediment yeast formed was placed on gypsum blocks. The chief experiments were made partly with *Sacch. Pastorianus I.* at 32° C., and partly with *Johannisberg II.* at 36° C. As an example of the result of the treatment of the first-named yeast at the different stages the following table is given:—

In the 2nd stage	1 per cent.	permanent	asporogenous	cells	were	found.
„ 4th „	60	„	„	„	„	„	„
„ 7th „	100	„	„	„	„	„	„

But besides these permanent asporogenous cells, other transition forms were found, *i.e.*, some which had only lost for a time the power of sporulating; after culture for a shorter or longer time in wort they again became sporogenous. It requires cultivation for two years before it can be decided whether a vegetation is a permanent asporogenous one or not.

Whilst nutrient liquids were employed in the foregoing experiments, Hansen also carried out numerous experiments with solid substrata as the cultivating medium.

Sacch. cerevisiae I. and *Sacch. Pastorianus II.* formed a considerable number of permanent asporogenous cells on wort gelatine at 25° C.: it further became evident that *Sacch. Pastorianus III.*, *Sacch. ellipsoideus II.* and *Johannisberg II.* formed very few, and finally that *Sacch. Pastorianus I.*, *Sacch. ellipsoideus I.*, *Sacch. Ludwigii* and *Sacch. membranaefaciens* formed no permanent asporogenous cells whatever. A permanent asporogenous variety was formed by *Sacch. anomalus* on agar-wort gelatine at 34° C. and on the same substratum at 32° C. by *Sacch. Pastorianus I.*

In order to decide the fundamental question whether, in the formation of these asporogenous varieties, it was a case of selection or transformation, Hansen made special experiments with the *Johannisberg II.* yeast. In a normal growth it was absolutely impossible to find a single cell which by normal culture did not produce a sporogenous growth. The starting point was a single cell, either a vegetative cell or a spore. The vegetation with which the experiment had been begun was analysed in such a way that at least 1,000 cells were isolated and the vegetations produced by them tested for spore formation. In all cases they sporulated in abundance. Hansen's experiments showed further that, as soon as the treatment is commenced, the transition forms—the temporarily asporogenous forms—appear. But such were never found at the starting point, and their inception must be ascribed to the treatment. Finally, the variation described is a general phenomenon which always makes its appearance if the cells are subjected to the above treatment. As a result, both of the analyses at the starting point and of those at the different stages of the treatment, it is plainly to be inferred that the variation which appears during the treatment originates in a transformation. The following behaviour, which is also treated experimentally here, is extremely peculiar, *viz.*, that even when the starting point of the experiment is a single vegetative cell or a spore, yet the following three categories appear during the treatment: sporogenous cells, temporary asporogenous cells and permanent asporogenous cells; a single cell can be again taken from the first two kinds which will once more reproduce all three categories.

As regards the conditions for transformation the following factors come to be considered: the chemical composition of the culture liquid, the vibrations caused by shaking the flask, the aëration of the culture liquid and the temperature.

The following can be set down as the result of Hansen's experiments: a culture liquid of definite chemical composition is as little required as are vibrations. Also aëration without the high temperature is incapable of causing the transformation. But it may be said of the culture liquid, the vibrations and the aëration, that each may have an indirect significance in so far as they all more or less aid multiplication. But a high temperature was shown to be the most important and absolutely essential factor. On culture gelatine we are able to produce the transformations described, not only by means of high temperature, but also by means of chemical factors.

Hansen has prepared asporogenous varieties from numerous other species besides those of *Sacch. Pastorianus I.* and *Johannisberg II.* The oldest of these are more than twelve years old, and they have always maintained their permanent asporogenous character in spite of numerous cultivations and very varied conditions. As a rule the saccharomycetes treated in the above manner lose simultaneously the power of forming spores and films. In some, also, a multiplying power of the cells in wort has been observed greater than that of the parent forms, and with regard to alcohol production greater deviations from the parent forms have been presented. In this respect Hansen has also brought about differences by other methods, obtaining both an increased and also a diminished production of alcohol. Thus Carlsberg bottom yeast No. 2 was cultivated at 32° C. in eight successive wort cultures, the succeeding one being inoculated from the preceding without any aëration of the flasks taking place. The ninth culture then, by cultivation in wort containing 10 per cent. of saccharose, gave 1 to 2 vol. per cent. less alcohol than the normal Carlsberg bottom yeast No. 1, and, in addition, also a better clarification in practice. Hansen obtained from spores of *Sacch. cerevisiæ I.* which were seeded on yeast water gelatine, a vegetation which gave up to 3 vol. per cent. more alcohol than the corresponding vegetation which had been cultivated only in beer wort during the whole time. In the latter case, however, it is selection rather than transformation which takes place. The single individuals of a species purely cultivated in wort may, *e.g.*, show a great difference in fermenting power, even although they have been produced in the same nutrient solution and under the same favourable conditions. These matters remain, for the most part, still in the dark ; the same remark applies also to variation as regards clarifying power.

If the culture of a sporeless non-film-forming variety be allowed to remain for a long time in the fermented nutrient solution, the latter exhibits an alcohol content far greater than that of the corresponding film-forming parent form reared under the same conditions. Thus, Hansen found that, in six months, there were 5·5 vol. per cent. of alcohol in the nutrient liquid of the variety, while there was only 1·5 vol. per cent. in that of the corresponding parent form, although the last-named flask also contained 5·5 vol. per cent. when it was only a month old. The cause lies in the fact already mentioned that film cells are capable of turning the alcohol formed into carbonic acid and water; the varieties here mentioned have lost this power of transforming alcohol simultaneously with the loss of the power of film formation.

With regard to these varieties which have lost their power of forming spores and films, it was found that these new properties are quite permanent and heritable. Such varieties have been cultivated under the most widely varied circumstances for long times (up to twelve years) without exhibiting the slightest indication of returning to the parent forms.

The number of transient variations is legion and they are easily produced. But of those which lead to permanent varieties the above is the only one we know of. The greatest interest attaches to it for several reasons. In the constant varieties mentioned the most important factor in transformation is temperature; but in order that the transformation may take place, a certain number of generations must be subjected to its influence. Since the same factors are at hand in nature as well as in the laboratory, the same variation may appear there also.

Practical Results and Variation in Practice.—If it is asked what practical utility such investigations can have, the following answer may be given: they deepen our insight into the life and the efficiency of these organisms, and we can therefore employ them in our service to a fuller extent than hitherto. We shall not be content as before to make our choice from among the species and varieties already known in nature or in the brewery, we shall rather begin ourselves to introduce improvements and transform species according to our desire.

The practical application to the analysis of brewery yeast consists in being able to make such an analysis with greater ease and certainty than formerly. One of our most important researches on wild yeast is based on spore culture, and species, as for example Carlsberg bottom yeast No. 1, which gives extremely few spores or none at all, are, in this respect, easy to control. If, on the other hand, a brewery yeast with strong spore formation has to be dealt with, the matter is somewhat more difficult. From the above it remains that we are able to take from the cells their power of forming spores, thereby making the analysis easier. Hansen has done this several years ago for Carlsberg bottom yeast No. 2, and has brewed good normal beer with the new variety formed in this manner. Yet it must be kept in mind that the transformed species may also undergo a simultaneous change in other respects. The sporeless variety will often act in a brewery in a different manner to its parent form. The experiments carried out with Carlsberg bottom yeast No. 1 are additional examples of the practical application of the results of the researches on variation. This yeast gives a beer that is stable, but greatly attenuated during the primary fermentation. Now Hansen has, by the method of culture described, prepared from it at a high temperature a permanent variety with less attenuating power which gives a fuller beer than the parent form. This variety has, however, one failing; it acts too slowly.

Numerous articles on the variation of beer yeast are to be found in the brewery journals both before and after the introduction of the pure culture system; the most of these treat of their degeneration in practice, others of their improvement. The attainment of the latter was sought in one way by their cultivation in any culture liquid of definite chemical composition. The best known researches in this direction are those made by Hayduck. According to him the enrichment of the yeast with nitrogen is one of the causes of degeneration; in such cases, in order to regenerate the yeast, he recommends that it should first, before pitching, be allowed to ferment in a cane sugar solution. Seyffert found recently that a yeast which had hitherto given a good clarification in the brewery, but which suddenly went bad in that respect, could be brought round again to give a good clarification by the addition of gypsum to the brewing water. The experiments with stimulating antiseptics already mentioned made by Biernacki, Effront, Hayduck, Heinzelmann and Schulz come under this head. Besides Hansen, Delbrück, Jörgensen, Kukla and Will have published papers on the variation of brewery yeast. Hansen's two new varieties of beer yeast and his experiments with them in practice have been described. These experiments assume a special interest from the fact that the two species which formed the starting points for the transformation are well known, and are present in most laboratories, and also from the fact that a definite method is given. This does not hold for the experiments described below which start with the object of attaining a racial improvement by selection in seeding.

The application of the pure culture system in practice consists, as we have seen, not only in the preparation of pure cultures of a certain species or race, but at the same time in a selection from among the vegetations produced by the individuals. Thus with the introduction of the pure culture system into the brewery a racial improvement is at the same time striven after. It was a case here of always selecting the best, since practical men always made greater demands and forced us to experiment, *i.e.*, compelled us to seek for such individuals as satisfied these demands. It is not only required that the species or race shall keep all those properties which are of value for the practical man, but it is desired that at the same time such individuals shall be selected as will vary in a manner serviceable to the brewery concerned, *i.e.*, possess good qualities in a high degree and lose undesirable ones. Of course even in the best cases this may be only partially attained. The yeast species selected by Hansen for the two Carlsberg breweries was the one which became known as Carlsberg bottom yeast No. 1, which has its good qualities (good taste, great stability), but also its less desirable ones (sluggish clarification, somewhat too great attenuation). He then began at once to improve the race by continual selection, and his successors have, later on, worked in the same direction, outside the Carlsberg laboratory as well, not only with this species but with other species and races.

Racial improvement thus consists in repeated selection of the best individuals. It may be seen from the papers published by Hansen and other investigators on experiments in this direction that it is not possible to set up definite rules here. Experiments must be carried out. The experimenter will often be deceived and arrive at an undesirable result; he is here confronted by something which is not to be regulated. It is a matter quite different from the mere preparation of the above asporogenous races; in the latter case the conditions are known exactly and the transformation can be regulated. Finally it must be borne in mind that, even when the material for such experiments on racial improvement is taken from the contents of the brewery fermenting vats, there is no guarantee that the race taken out stands in genetic connection with that formerly selected. They are not therefore of necessity blood relations because both agree in botanical characteristics.

Similar communications on racial improvement have been made by the technologists of wine fermentation, but here also there is no mention made of definite methods. In all these communications also, information about the species from which the start was made is as a rule wanting.

A culture yeast may also be subject in practice to variation in a harmful direction. Hansen in his *Practical Studies in Fermentation,* when in 1892 summing up his experiments on this subject during the course of years, says as follows: "When we regard the variations of yeast in brewery practice from a biological point of view, we are inclined to look upon them as quite insignificant; for the practical brewer, however, the matter is quite different. The changes can, indeed, occur in a very disagreeable manner, and sometimes cause an appreciable irregularity. In the course of a year they pass like a wave through the brewery, and in most cases we have no idea of their cause." Investigations on all that occurs in practice are difficult in a high degree, not only because we do not work here with the absolutely pure culture of the culture yeast concerned, but the composition of the wort and other external factors are so very complicated and variable that they frequently escape our control. In spite of very extended and arduous attempts on the part of the author by experimental means to shed light on the most important questions which have cropped up in this region of late years, he is still unable to record completely satisfactory results, but hopes later to be able to give some elucidations. This may, however, be said, that those variations which come to light in actual practice under the influence of the factors there predominant are only transient, which shows that the pure culture system has not only gained a firm foothold in the brewing system of the whole world, but daily spreads further into all the other alcohol fermentation industries. Some of the culture yeasts are particularly permanent, whilst others are more inclined to variation. Carlsberg bottom yeast No. 1 belongs to the first of these. In the New Carlsberg Brewery, for instance, there was a pure culture of this species in the fermentation cylinder of the pure culture apparatus which had been introduced more than five years before. Fluctuations of course occurred, but no fixed changes. Various authors have made communications on a special constancy in culture yeasts. The experiments on this point by Irmisch, Jörgensen and P. Lindner are noteworthy. Finally, if in practice a yeast growth is present which exhibits a variation in an adverse direction, a degeneration, the means of cure are now easily accessible; a new culture is then introduced.

10.—*Circulation in Nature.*

We will now shortly describe what is so far known of the circulation of the saccharomycetes in nature.

In the years 1880-81 Hansen published the results of one of the most important and most interesting biological researches on yeast cells which we possess, and which he had carried out in the preceding years with the cells of the yeast named *Saccharomyces apiculatus*, Reess. From it we find that this fungus occurs and fructifies in summer and autumn on the injured parts of sweet juicy fruits, in the juice of which it multiplies; during winter and spring it is found in the ground under the fruit and only in quite exceptional cases in other places. It gets into the ground partly through the falling of the fruit and partly by means of the rain which trickles over the fruit. Those cells which pass into the intestines of birds and insects and are given off with the excrements also pass finally into the earth.

Hansen has likewise shown that *Sacch. apiculatus* appears normally only on sweet juicy fruits and under the latter in the ground; the cause of this is the very slight power of resisting drying which this fungus possesses. Therefore, when present on the surface of unripe whole fruit, on leaves, twigs, etc., where it cannot multiply, it dies off after a comparatively short time through drying. Otherwise it maintains its life in fruit juice where it multiplies, and in the soil where it is protected from drying. Direct experiments have demonstrated this.

From its winter abode in the ground it again reaches the sweet juicy fruit by aid of the wind, and of insects and other small animals. Rain may also be effectual in this respect, *viz.*, in the case of fruit growing near the ground. As soon as multiplication goes on in the fruit juice, insects again play an important part in distribution by carrying the cells from fruit to fruit; according to Wortmann wasps are particularly efficacious in this respect in vineyards. Insects are, however, the distributing agents only during a small part of the year, and then only on the sunny days of that period; all through the year the wind carries the cells about with the dust and finally deposits them in large quantities on the fruit.

With regard to typical saccharomycetes recent investigations of Hansen have shown that they likewise pass the winter in the ground, and that sweet juicy fruits are essential breeding places for them, so that they pass through the same circulation in nature as *Sacch. apiculatus.* The investigations of Müller-Thurgau and Wortmann confirm this. Pasteur was of the opinion that wine yeasts do not spend the winter underground. Hansen's experiments, however, speak against this, since he found living yeast in the soil under the vines in the wine-producing districts of Germany in spring and summer, *i.e.*, at a time when there are no ripe grapes. Hansen has, besides, seeded saccharomycetes in the soil under natural conditions, and has found them living there more than three years after. After finding that the breeding places of saccharomycetes are on sweet juicy fruit he proceeded from this and followed the cells farther on their way during the different seasons of the year. The saccharomycetes may of course follow, besides the normal circulation, some other accidental and exceptional one. Only the regular annual repetition of the circulation is of interest in relation to a knowledge of this portion of the economy of nature.

Some writers have expressed the view that the breeding places of the saccharomycetes are in the nectar of flowers until the time of ripe fruit, and that the winter is passed in the intestines and excrements of insects and other animals. Hansen's investigations have shown, however, that if they are found in these places at all, it is quite a matter of accident. The author's experiments on insects have also demonstrated this.

Hansen's researches on the circulation of saccharomycetes in nature, and on the amount of micro-organisms in the air at various seasons of the year, have led to the following results, very important to the brewer: (1) Wind and insects are the most important means of transportation of yeast cells in nature, especially the first; (2) dust clouds in the harvest months are rich in strong yeast cells, produced on sweet juicy fruit; and (3) all through the year the open cooling vessels are the chief means by which wild yeast species make their way into the brewery.

The practical outcome of this was that, in the Old and New Carlsberg Breweries in Copenhagen, the ways in the neighbourhood of the cooling vessels were sprinkled with water, so that the dust might not infect the wort, a procedure which was followed in other places. Later, after the pure culture system was introduced, the open cooling vessels were removed from the above breweries and replaced by closed holders for cooling and aërating the wort.

The cask deposit and likewise impure pitching yeast may also give rise to infection; before introducing the pure culture system the latter was probably the worst source of infection. If, by any means, wild yeast, etc., makes its way into practice, it may collect in the water pipes, especially at the connections, and these are, therefore, according to Will's experiments, the most dangerous sources of infection if the wort has been once infected; the cracks and crevices in fermenting vessels are also dangerous in this respect. It is therefore very important to keep these places clean. Will's experiments have also clearly shown that the filter bag is a source of danger. But the wind with the dust it carries remains, all the year round, the chief means by which uninvited intruders make their way into the brewery. Combined with this we have the insects in the summer months on sunny days. Now that impure pitching yeast has become rare, the open cooling vessels therefore form the most important and most dangerous source of infection.

SYSTEMATIC.

1. *Genus: Saccharomyces*, (*Meyen*) *Reess.*

(Synonyms: Mycoderma, Persoon; Cryptococcus, Kützing; Torula, Turpin; Hormiscium, Bail.)

Single cell fungi in which vegetative increase takes place by budding and which develop endospores in their interior under certain conditions. Sometimes they may form a typical mycelium.

The various related species here may, as before stated, be grouped according to their action on the various sugars. The following classification is of this kind, and its basis is to be found in Hansen's paper of 1888:—

1. Those species which ferment maltose, dextrose and saccharose.

Examples:	*Sacch. cerevisiae I.,*	**Hansen.**
	,, *Pastorianus I.,*	,,
	,, ,, *II.,*	,,
	,, ,, *III.,*	,,
	,, *ellipsoideus I.,*	,,
	,, ,, *II.,*	,,

besides brewery yeast species and in general the culture yeasts hitherto investigated.

2. Those species which ferment dextrose and saccharose but not maltose.

Examples:	*Sacch. Marxianus,*	**Hansen.**
	,, *exiguus* (Reess),	,,
	,, *Ludwigii,*	,,
	,, *Saturnus,*	**Klöcker.**

3. Those species which ferment dextrose, but neither saccharose nor maltose.

Example: *Sacch. mali Duclauxi*, Kayser.

4. Those species which ferment dextrose and maltose, but not saccharose.

Example: *Sacch n. sp.*, isolated from the stomach of a bee by the author.

5. Those species which ferment neither maltose, dextrose nor saccharose.

Examples:	*Sacch. membranaefaciens,*	**Hansen.**
	,, *hyalosporus,*	Lindner.
	,, *farinosus,*	,,
	,, *anomalus var. belgicus,*	,,

6. Those species which can ferment lactose.

Example: *Sacch. fragilis*, Jörgensen.

The descriptions and investigations of the species given below are from the authors who have established the species in question, and whose names are added to the systematic name of the species. Where the researches of other investigators are given this is specially mentioned.

accharomyces cerevisiæ I., Hansen (Figs. 79 1, 80, 81, 82, 8 and 89), was isolated from a top yeast in an Edinburgh very, and found later also in the yeast of a London very. The cells in the yeast sediment are, as a rule, e and round (Fig. 87). In film growths at 6° to 15° they are for the most part of the same shape as in the iment yeast, with only isolated abnormal forms (Fig. 88). e size of the spores varies from 2½ to 6 μ; there e usually one to four in each cell, seldom five (Figs. 1 and 89). The germination of the spores is represented Figs. 80, 81 and 82.

At 37½° C. no spores develop.
At 36° to 37° C. the first indications are seen after 29 hours.
At 30° C. " " " " " 20 "
At 11° to 12° C. " " " " " 10 days.
At 9° C. no spores develop.

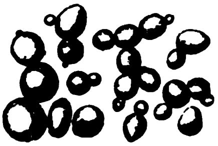

FIG. 87.—*Saccharomyces cerevisiæ I.*, Hansen. Sediment yeast. $\frac{1000}{1}$. (After Hansen.)

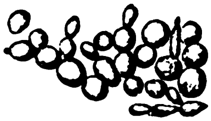

FIG. 88.—*Saccharomyces cerevisiæ I.*, Hansen. Film growth at 15·6° C. $\frac{1000}{1}$. (After Hansen.)

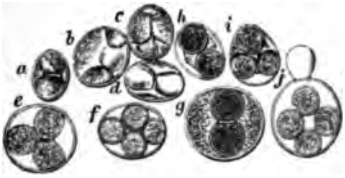

FIG. 89.—*Saccharomyces cerevisiæ I.*, Hansen. First stages of development of the spores. $\frac{1000}{1}$. (After Hansen.)

The temperature limits for film formation are 33° to 34° C. and 6° to 7° C. The species is a vigorous top beer yeast.

We shall describe some of the numerous forms which are employed in the industry.

The cell form of **Carlsberg Bottom Yeast No. 1, Hansen,** may be seen from Fig. 90. The species forms spores with extreme difficulty; after a long time (five to six days at 25° C.) they are only to be found singly. Very often there are none at all. In practice it is inclined to give only an indifferent clarification and a strong attenuation, but, on the other hand, produces a fine stable beer.

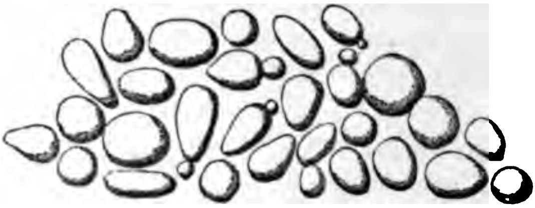

FIG. 90.—*Carlsberg Bottom Yeast No.* 1, Hansen. $\frac{1000}{1}$. (After Hansen.)

Carlsberg Bottom Yeast No. 2, Hansen.—As seen in Fig. 91, the shape of the cells is more regular than in the preceding species; it also forms spores somewhat more easily. Beer prepared with this yeast is not so stable, but clears better in practice.

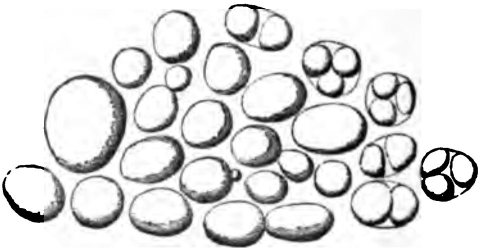

FIG. 91.—*Carlsberg Bottom Yeast No.* 2, Hansen. Some cells with spores. $\frac{1000}{1}$. (After Hansen.)

Of four culture yeasts of the Munich station described by Will, **Tribe 93** and **Tribe 2** belong to the strongly fermenting species, **Tribe 6** to the yeasts of medium fermentation, while **Tribe 7** is to be classed with the feebly fermenting species. The last forms spores with great difficulty, while first three produce them abundantly and easily. The dinal points for spore and film formation are the lowing:—

	Tribe 2.	Tribe 6.	Triba 93.	Tribe 7.
mits for spore formation.	31° to 11° C.	31° to 11° C.	30° to 10° C.	30° to 13° C
ptimum for spore formation.	25° to 26° C.	28° C.	28° C.	25° to 26° C.
mits for film formation.	28° to 31° 7° to 10° C.	25° to 31° 7° to 10° C.	30° to 31° 4° to 7° C.	25° to 28° 4° to 7° C.

Film formation goes on most quickly in Tribe 7. The cells of all four species are round or oval; in Tribe 7 giant cells occur regularly.

The three types of brewery yeasts, Saaz, Frohberg and Logos, have been mentioned on p. 219 with regard to their action on the sugars.

There is also a very large number of **Beer Top Yeast Species** and **Races**. Hansen, in his paper of 1882, gives a description of two such species prepared by pure culture; the one was isolated from a Burton yeast, the other from an Edinburgh one. They were plainly different from one another. A detailed description of Hansen's top yeast, *Sacch. cerevisiæ I.*, has already been given. Several others have been prepared by pure culture later, especially by Jörgensen and Schönfeld. Jörgensen groups the forms present in his institute, including the bottom yeast species as well, from the practical point of view, *viz.*, according to how quickly or slowly they clear and at the same time ferment strongly or weakly.

The distillery yeast, **Race II.**, isolated at the Berlin Station, has attained a wide distribution in Germany; it originated in a distillery in West Prussia, is a top yeast of the Frohberg type, and is distinguished chiefly by its large cells and its great fermentative power. The results obtained from it in practice are very good; it is especially adapted for the fermentation of difficultly fermentable and highly concentrated mashes, its power of resistance to high alcoholic content being great.

The station at Berlin has also isolated various yeasts for use in pressed yeast manufacture, and Race IX. especially has given satisfactory results.

We shall now describe several wild yeasts.

Saccharomyces Pastorianus I., Hansen (Figs. 79₃, 92 and 93), was first found in dust in the air of a Copenhagen brewery, and later also in diseased beer. The growth in wort consists chiefly of sausage-shaped cells, but round and oval cells are also found (Fig. 92). The size of the spores is 1½ to 3½ μ, they seldom attain a diameter of 5 μ. They number oftenest 1 to 4, sometimes also 5 to 10 in very elongated cells.

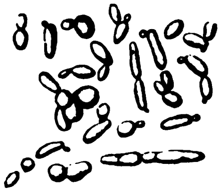

Fig. 92.—*Saccharomyces Pastorianus I.*, Hansen. Sediment yeast. $\frac{500}{1}$. (After Hansen.)

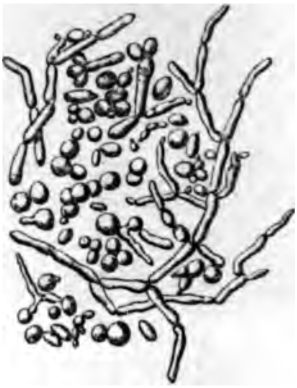

Fig. 93.—*Saccharomyces Pastorianus I.*, Hansen. Film growth at 15—3° C. $\frac{500}{1}$. (After Holm in Hansen's paper.)

At 31½° C. no spores develop.
,, 29½° to 30½° C. the first indications are seen after 30 hours.
,, 27½° C. ,, ,, ,, ,, ,, 24 ,,
,, 3° to 4° C. ,, ,, ,, ,, ,, 13 days.
,, ½° C. no spores develop.

The temperature limits for film formation are 26° to 28° C. and 3° to 5° C. This species is a bottom yeast form and, as already stated, a dangerous disease yeast in breweries, since it causes a disagreeable smell and a strong bitter taste in the beer. It usually has a detrimental effect on the clarification as well. While thus possessing bad qualities for beer manufacture, it can yet give a good product in the preparation of wine (Mach and Portele).

Saccharomyces Pastorianus II., Hansen (Figs. 79 3, 94 and 95), was also found by Hansen in the air in a Copenhagen brewery. The cells are usually somewhat larger than those of the preceding species. This yeast produces a feeble top fermentation. The size of the spores is 2 to 5 μ, seldom 4 to 5 μ.

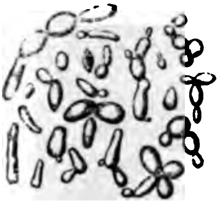

FIG. 94.—*Saccharomyces Pastorianus II.*, Hansen. Sediment yeast. $\frac{1000}{1}$. (After Hansen.)

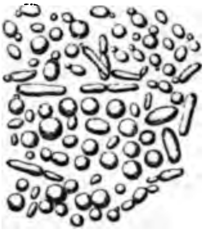

FIG. 95.—*Saccharomyces Pastorianus II.*, Hansen. Film growth at 15—3° C. $\frac{1000}{1}$. (After Holm in Hansen's paper.)

At 29° C. no spores develop.
,, 27° to 28° C. the first indications are seen after 34 hours.
,, 25° C. ,, ,, ,, ,, ,, 25 ,,
,, 3° to 4° C. ,, ,, ,, ,, ,, 17 days.
,, ½° C. no spores develop.

The temperature limits for film formation are 26° to 28° C. and 3° to 5° C. The cells of the young film at 13° to 15° C. are distinguished from the corresponding cells of the succeeding species by generally being round or oval, whereas in *Sacch. Pastorianus III.* many sausage-shaped cells are found under these conditions. Streak cultures on yeast-water gelatine at 15° C. show colonies with smooth borders in sixteen days, the species differing from *Sacch. Pastorianus III.* in this respect also.

Saccharomyces Pastorianus III., Hansen (Figs. 79 4, 96 and 97). This species was found in bottom fermentation Copenhagen beer affected with yeast turbidity. The shape of the cells is, for a culture in wort, the same as those of the two preceding species. The spores are 2 to 4 μ in size, seldom 3½ to 4 μ.

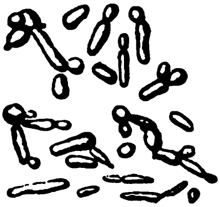

FIG. 96.—*Saccharomyces Pastorianus III.*, Hansen. Sediment yeast. $\frac{1000}{1}$. (After Hansen.)

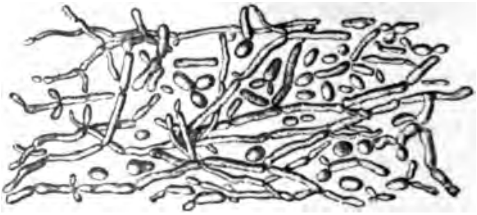

FIG. 97.—*Saccharomyces Pastorianus III.*, Hansen. Film growth at 15-3° C. $\frac{200}{1}$. (After Hansen).

At 29° C. no spores develop.
„ 27° to 28° C. the first indications are seen after 35 hours.
„ 25° C. „ „ „ „ „ 28 „
„ 8½° C. „ „ „ „ „ 9 days.
„ 4° C. no spores develop.

The temperature limits for film formation are 26° to 28° C. and 3° to 5° C. The cells of the young film at 13° to 15° C. are distinguished from the corresponding cells of *Sacch. Pastorianus II.* by many of them being very long and ge-shaped (Fig. 97) ; in the latter species the cells are ently round or oval.

treak cultures on yeast-water gelatine at 15° C. give, kteen days, colonies with distinctly hairy borders.

This species produces a more vigorous top fermentation *Sacch. Pastorianus II.* It is a dangerous disease yeast ch causes turbidity in beer. But a small addition of this species to stock yeast can, in some circumstances, make opalescent beer clear, probably by removing during after-fermentation the substances which cause the opalescence.

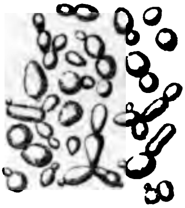

FIG. 98.—*Saccharomyces ellipsoideus I.*, Hansen. Sediment yeast. $\frac{1000}{1}$. (After Hansen.)

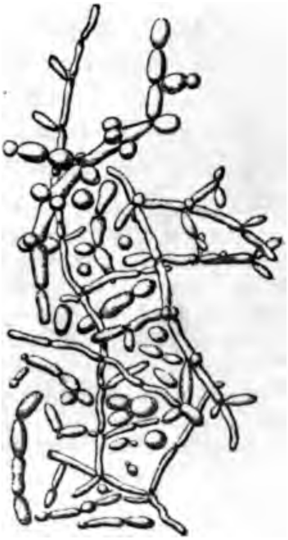

FIG. 99.—*Saccharomyces ellipsoideus I.*, Hansen. Film growth at 15-13° C. $\frac{1000}{1}$. (After Holm in Hansen's paper.)

Saccharomyces ellipsoideus I., Hansen (Figs. 79 *b*, 98 and 99).—This species was found by Hansen on the surface of ripe grapes in the Vosges district. The cells are of ellipsoidal shape, but may also be sausage shaped. The spores are 2 to 4 μ in size, seldom 3½ to 4 μ.

At 32½° C. no spores develop.
„ 30½° to 31½° C. the first indications are seen after 36 hours.
„ 25° C. „ „ „ „ „ 21 „
„ 7½° C. „ „ „ „ „ 11 days.
„ 4° C. no spores develop.

The temperature limits of film formation are 33° to 34° C. and 6° to 7° C. The cells of the young film at 13° to 15° C. are distinguished from the corresponding ones of *Sacch. ellipsoideus II.*, which are round or oval, by the large number of long, sausage-shaped cells (Fig. 99).

Streak cultures on wort gelatine at 25° C. after eleven to fourteen days give colonies with a peculiar reticulated structure (here differing from Hansen's foregoing species and from *Sacch. ellipsoideus II.*).

This species is one of the many which are active in wine fermentation. Numerous forms, closely related to this species, have been isolated in the experimental stations for wine culture by Aderhold, Hotter, Marx, Müller-Thurgau, W. Seifert, Wortmann and others. Among these species there are some in which the cells are vigorous spore formers, *e.g.*, the species "*Johannisberg II.*," which has become so well known through Aderhold and Wortmann's researches, and of which 99 to 100 per cent. of the cells develop spores on gypsum blocks. The maximum temperature for spore formation is, in this species, according to Hansen, between 33° and 34½° C., and the minimum temperature between 3° and 2° C. Another wine yeast is the *Walporzheim* yeast. According to Aderhold this yeast is distinguished, in one respect, by the rapidity with which it forms a film in which a large quantity of spore-bearing cells appear.

Saccharomyces ellipsoideus II., Hansen (Figs. 79$_6$, 100 and 101), is a very dangerous disease yeast (yeast turbidity) in bottom fermentation breweries. The spores are 2 to 5 μ in size, seldom 4 to 5 μ.

At 35° C. no spores develop.
„ 33° to 34° C. the first indications are seen after 31 hours.
„ 29° C. „ „ „ „ „ 22 „
„ 8° C. „ „ „ „ „ 9 days.
„ 4° C. no spores develop.

The temperature limits for film formation are 36° to 38° C., and 3° to 5° C.

The cells of the young film at 13° to 15° C. are distinguished from the corresponding ones of *Sacch. ellipsoideus I.* by being chiefly round or oval in shape.

Two disease yeasts isolated by Will are very closely related to this species. The cardinal points in the spore formation of these two yeasts are as follows: For one:—

At 41° C. no spores develop.
,, 39° C. the first indications are seen after 23 hours.
,, 34° C. ,, ,, ,, ,, ,, 11 hours.
,, 8° to 9° C. ,, ,, ,, ,, 9 days.
,, 4° to 5° C. no spores develop.

And for the other:—

At 32° C. no spores develop.
,, 30° to 31° C. the first indications are seen after 48 hours.
,, 23·5° to 24° C. ,, ,, ,, ,, ,, 29 ,,
,, 3° C. ,, ,, ,, ,, ,, 21 days.
,, 0·5° to 1° C. no spores develop.

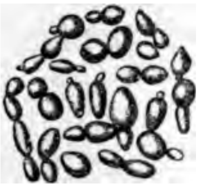

FIG. 100.—*Saccharomyces ellipsoideus II.*, Hansen. Sediment Yeast. $\frac{500}{1}$. (After Hansen.)

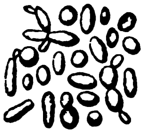

FIG. 101.—*Saccharomyces ellipsoideus II.*, Hansen. Film Growth at 28° to 3° C. $\frac{500}{1}$. (After Hansen.)

Saccharomyces Ilicis, Grönlund, was found on the fruit of *Ilex Aquifolium.* The cells are mostly spherical in shape. The cardinal points for spore formation are the following:—

At 38° C. no spores develop.
,, 36° to 37° C. the first indications are seen after 22 hours.
,, 32° C. ,, ,, ,, ,, ,, 18 ,,
,, 9½° C. ,, ,, ,, ,, ,, 20 days.
,, 8° C. no spores develop.

Streak cultures on wort gelatine have a mealy appearance. Wort fermented with this species assumes a disagreeable bitter taste. It is a bottom yeast which ferments saccharose, dextrose and maltose; in wort it produces 2·78 vol. per cent. of alcohol.

Saccharomyces Aquifolii, Grönlund, was discovered on the same fruit. It is a top yeast and, judging from the appearance of the spores, a culture yeast. The cardinal points for spore formation are the following:—

At 30½° to 31° C. no spores develop.
,, 27½° to 28½° C. the first indications are seen after 29 hours.
,, 27° C. ,, ,, ,, ,, ,, 28 ,,
,, 10° to 10½° C. ,, ,, ,, ,, ,, 15 days.
,, 8° to 8½° C. no spores develop.

Streak cultures on wort gelatine have a shiny appearance. This species gives to wort a sweet taste with a bitter after-taste and ferments saccharose, dextrose and maltose. In wort it forms 3·71 vol. per cent. of alcohol.

Saccharomyces Vordermanni, Went and Prinsen Geerligs, is, in appearance, similar to wine yeast. The cells are rounded like a pear or onion; sometimes angular or elongated cells are found. The number of spores is usually four; a film is not formed. Like all the preceding species it ferments maltose, dextrose and saccharose, the latter after inversion. It forms 9 to 10 per cent. of alcohol. The fungus is present in "Raggi," which is employed in Java in the manufacture of arrack.

"Raggi" is made in the form of balls or cakes which consist of rice, pieces of sugar cane and other vegetable substances, and are saturated with organisms. Among the latter are bacteria, yeast cells and mould fungi. The presence of the first of these is not advantageous to fermentation, but rather unfavourable if they are in large quantity. The yeast cells belong partly to *Sacch. Vordermanni* and partly to *Monilia javanica*. The mould fungi are the *Mucor oryzæ* and *Rhizopus oryzæ* already described; they both turn rice starch into sugar, which is then fermented both by *Sacch. Vordermanni* and *Monilia javanica*. The first of these two fungi gives a very fine arrack, whilst the latter produces an alcohol with a bad taste. Here, therefore, a pure culture of *Sacch. Vordermanni* could be employed to great advantage.

Saccharomyces pyriformis, Marshall Ward, is active in the fermentation of ginger beer. The cells are similar in hape to those of the *Sacch. ellipsoideus* group. On wort it forms a film composed of pear or sausage-shaped cells. In conjunction with the *Bacterium vermiforme*, also found by Marshall Ward, it produces, from a sugar solution containing ginger, an acid frothing beverage, ginger beer, which is prepared in many cottages in England.

Saccharomyces Marxianus, Hansen, was found on grapes. The cells are small, oval or egg shaped, or elongated and sausage shaped, often in colonies. When cultures have been in wort for some time, bodies form which consist of mycelium-like colonies. When the wort cultures are two to three months old a film is just visible, consisting partly of short, sausage-shaped cells and partly of oval ones. On solid substrata Hansen observed that this species develops a mycelium which is similar, for instance, to that formed by *Monilia candida*. The spores are more or less kidney shaped, sometimes, however, round or oval, and usually about 3·5 μ long. After cultivation in wort they are not formed particularly easily, but are formed much more quickly and in large quantity after cultivation in a nutrient solution containing dextrose (the author).

The cardinal points for spore formation are, according to the author, as follows: maximum between 34° and 32° C., optimum between 25° and 22° C., and minimum between 8° and 4° C.

According to Hansen it gives only 1 to 1·3 vol. per cent. of alcohol after standing for a long time in wort. It is capable of fermenting dextrose and saccharose, the latter after inversion, but not maltose. In a 15 per cent. solution of saccharose in yeast water 3·75 vol. per cent. of alcohol were formed at 25° C. in eighteen days, and 7 vol. per cent. in thirty-eight days. In yeast water containing 10 and

15 per cent. of dextrose it formed, in a month, 6·5 and 9 vol. per cent. of alcohol respectively.

Saccharomyces exiguus, (Reess) Hansen, was found in general in pressed yeast. This species is distinguished from the preceding one in that it does not develop mycelium-like colonies in wort nor mycelia on gelatine. The formation of spores is scanty; here also only the semblance of a film is formed after several months. Like the preceding species it is incapable of fermenting maltose. It formed 6 vol. per cent. of alcohol in a 15 per cent. solution of saccharose in yeast water. In a 15 per cent. dextrose solution 8 vol. per cent. of alcohol were formed in fourteen days at 25° C.

Formerly the opinion was held that this species was the cause of turbidity in beer. Hansen has, however, shown that even a large addition of this fungus at the beginning or end of the primary fermentation or during storage causes no kind of disease in lager beer.

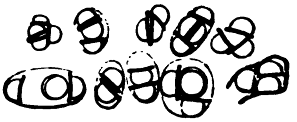

FIG. 102.—*Saccharomyces anomalus*, Hansen. Spore-bearing cells. $\frac{1000}{1}$. (After Hansen.)

Saccharomyces anomalus, Hansen (Figs. 83 and 102), was found by Hansen in an impure brewery yeast from Bavaria. This fungus was also found later in English and Belgian beer, on green malt, on bran, in syrup of althea, in soil and on fruit, *e.g.*, plums.

In wort it produces fermentation very soon, both at ordinary room temperature and at 25° C. At the very beginning of the fermentation a dull grey film is formed; during fermentation the liquor is turbid and a smell like fruit ether is developed. The microscopic appearance of the vegetative cells is reminiscent of a *Torula.* They are small, oval and sometimes sausage shaped; many of these are found particularly in old cultures. After some time cells with spores may be found both in the film and in the sediment yeast. Two to four spores are formed in each cell; these are distinguished from the spores of all other saccharomycetes by their shape. They are, in fact, hemispherical with a projecting rim round the edge of the flat side, *i.e.*, hat-shaped. The diameter of the flat side, not including the rim, is 2 to 3 μ.

Nielsen found the following cardinal points for spore development:—

At 34° C. no spores develop.
,, 32½° to 32° C. the first indications are seen after 19 to 21 hours.
,, 30° C. ,, ,, ,, ,, ,, 17 to 19 ,,
,, 7½° to 6° C. ,, ,, ,, ,, ,, 13 to 14 days.
,, 3° to 2½° C. no spores develop.

The shape possessed by the spores of this species is also found in those of *Endomyces decipiens*, a fungus which appears as a parasite on a mushroom (*Agaricus melleus*), on the lamellæ of which it spreads itself as a whitish layer. In spite of the similarity of the spores, however, *Endomyces decipiens* has no genetic connection with *Sacch. anomalus.* Further, the spores of the latter on germinating form buds, whilst those of *Endomyces* form germ threads; in this latter species no budding takes place at all.

According to Nielsen's experiments, *Sacch. anomalus* in wort forms 0·9 vol. per cent. of alcohol and ester in eleven days. W. Seifert states that the ester formed here is ethyl acetate, and that in wort containing alcohol the species forms acetic acid and decomposes the alcohol into carbonic acid and water; a prolonged action of the fungus destroys the acetic ester. According to Nielsen, it causes no fermentation in maltose solutions, and secretes hardly any invertase.

Related forms have been found by Holm, Jörgensen, Lindner, Will, Zeidler and the author. Lindner found one of this kind, among others in an Armenian beverage, "Mazun". Jörgensen states that he has found a related form in English top fermentation beer which had yeast turbidity. In bottom fermentation beer it has been observed several times, but never under such conditions that it could be looked upon as a disease yeast.

A species which also has hat-shaped spores has been named *Sacch. anomalus, var. belgicus*, by Lindner; it ferments neither maltose, dextrose nor saccharose, and produces no smell of fruit ester. Beijerinck describes a typical *Sacch. anomalus* under the name *Mycoderma pulverulenta*; others have also isolated new related species.

Saccharomyces membranæfaciens, Hansen, was found in a gelatinous mass which had developed on the injured roots of an elm tree. It was also found later by Koehler in the water from a polluted stream, and by Jörgensen in white wines.

The species quickly forms, on the whole surface of the wort, a strongly developed, light gray corrugated film, consisting chiefly of sausage-shaped and elongated, oval cells rich in vacuoles; wort gelatine is liquefied very quickly by it: it forms on this substratum dull gray colonies, often with a reddish tinge. They are similar to the colonies of *Mycoderma cerevisiæ* and *M. vini*.

The shape of the spores is very variable; they are often rounded and sometimes inclined to a hemispherical shape. They are formed in large quantity both on gypsum blocks and in films.

This species is distinguished by its inability to form alcohol; it produces fermentation neither in saccharose, dextrose, maltose nor lactose solutions; it also secretes no invertase.

Nielsen found that—

At 35° no spores developed.
" 33½° to 33° C. the first indications are seen after 19 to 21 hours.
" 31° to 30½° C. " " " " " 17 to 18 "
" 7½° to 6° C. " " " " " 6 to 7 days.
" 3° to 2½° C. no spores developed.

W. Seifert found that *Sacch. membranæfaciens* grows even in the presence of 12·2 vol. per cent. of alcohol. In an artificial nutrient liquid (Pasteur's solution) with 4·8 vol. per cent. of alcohol and an addition of malic acid it formed 0·110 per cent. of glycerine in fourteen weeks; during the same time 3·8 vol. per cent. of alcohol disappeared. It hardly attacks tartaric acid at all, nor citric acid, but malic acid. Acetic and succinic acids are completely consumed by it. The acetic acid and glycerine formed are again used up. In dextrose solutions (not in those of saccharose or maltose) it forms fixed and volatile acids. It destroys the bouquet of wine, the esters present being broken up, while new and less favourable ones are formed.

Seifert found in Crimean wine a variety which he calls *Sacch. membranæfaciens var. tauricus*. The latter stops growing in the presence of 12·2 vol. per cent. of alcohol. The maximum temperature for spore formation is 34° C., the minimum temperature 5° to 6° C.

Seifert also isolated another form from Californian claret, *viz.*, *Sacch. membranæfaciens var. californicus*, which is capable of growing in the presence of 12·2 vol. per cent. of alcohol. The temperature maximum for spore formation is 33° C., the minimum 7° to 12° C.

Both of the latter forms are further distinguished from *Sacch. membranæfaciens*, Hansen, by their forming much less glycerine in the above Pasteur solution and being able only to attack alcohol to a small extent.

Other related forms are described by Pichi under the names *Sacch. membranæfaciens II.* and *III.*, and by Lindner under the names *Sacch. hyalosporus* and *Sacch. farinosus.*

Saccharomyces Bailii, Lindner, was isolated from Danzig Jopen beer. It ferments dextrose, inverts saccharose, and is distinguished by its developing extremely remarkable amœbiform cells in old cultures. It forms no film.

Saccharomyces mali Duclauxi, Kayser, which was found in cider, is described as follows: The cells are 6 to 12 μ long and 4 to 7 μ broad and form an easily disturbed sediment. They are moderately sensitive to acids, and die off at about 55° C. Spores appear at 15° C. in thirty hours. This yeast ferments neither saccharose nor maltose, but ferments invert sugar and imparts a distinct bouquet to the fermented liquid.

Saccharomyces cartilaginosus, Lindner, was found in Kefir. The species ferments in wort and gives to it a somewhat smoky taste; like *Sacch. Pastorianus III.* it forms much frilled giant colonies and streak cultures. The plasma is peculiarly granular. On the surface of the wort after some weeks, small well-defined island colonies are formed, of somewhat compact, almost gristly consistency. Coalescence of the islands into one single layer does not take place. The sediment yeast is flocculent. In contradistinction to the following species it does not ferment milk sugar.

Saccharomyces fragilis, Jörgensen, was also found in Kefir. It ferments milk sugar. Jörgensen gives the following description: The growth consists of comparatively small oval and elongated cells. In cultures on gypsum blocks spore formation appears distinctly at 25° C. in twenty hours, at 15° C. in forty hours. The long, rounded shape of the spores is characteristic. These form both in fermenting liquids and on gelatine. In 10 per cent. lactose-yeast-water the species gave about 1 part per cent. by weight of alcohol at the room temperature in eight days; after four months it gave 4 parts per cent. by weight of alcohol. In hopped wort (about 11 per cent. Ball.) it produced about 1 part per cent. by weight of alcohol at room temperature in ten days.

By Kefir, as is well known, is understood the beverage which originated in the Caucasus and is prepared there by fermenting milk. This fermentation is brought about by the so-called Kefir grains, which are yellow, hard granules of the size of a pea. The mode of preparation in the Caucasus consists in placing the milk in goatskins to which the above corns are next added; it is then all shaken up from time to time. After a few days the drink is ready. The Kefir grains are taken out and kept for another fermentation. During fermentation, alcohol, lactic acid and carbonic acid are formed; these products are formed by the accidental co-existence[1] of various organisms of which the number and species are very variable. Freudenreich found, in the Kefir grains examined by him, a *Torula* and three bacteria, among these the *Bacillus caucasicus*. The last appears to be always present with an alcoholic yeast fungus. Besides the two saccharomycetes mentioned, several other species of *Saccharomyces* are cited as being found in Kefir.

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[1] In literature Kefir fermentation and other similar fermentations (*e.g.*, that of ginger beer) are represented and explained as the results of

In Armenia a beverage called Mazun, similar to Kefir, is prepared from milk. According to Emmerling the exciter of fermentation consists of a white, fatty, cheese-like mass which can be preserved for a long time. After a short time it produces alcoholic fermentation in the milk; simultaneously the casein coagulates, acid being formed, and a smell of fatty acid ester is developed. In this mass Emmerling found the following micro-organisms: yeasts among which were a number of coloured species, *Oidium lactis*, some other mould fungi, a yellow *Sarcina* and the common hay bacillus (*Bacillus subtilis*), also some cocci and the *Bacillus acidi lactici*, Hueppe. The last-named and the cocci turn the milk sugar into lactic acid; the milk sugar is also hydrolysed and thus rendered susceptible to the attacks of the yeasts. This fermentation is thus also the result of a fortuitous co-existence of bacteria and yeasts. As already mentioned, Lindner found *Sacch. anomalus* in this beverage.

Finally, we shall describe another species of *Saccharomyces* which was found in the exudations from oak trees, namely,

Saccharomyces Ludwigii, Hansen (Figs. 77, 84 and 85). —This species is the transitional form to the next genus, as with it something between a typical budding and a simple division takes place. It is in many respects so remarkable that it deserves a somewhat closer description. It is probably the only *Saccharomyces* which can be immediately recognised by microscopic examination. Although the vegetative cells possess all possible shapes, the lemon shape is the most prominent; it reminds one somewhat of that

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a symbiosis. This does not agree with the original signification of the word, since in the cases cited it is only a question of a casual co-existence, a mixed fermentation; otherwise every fermentation in which two or more species appeared would be a symbiotic fermentation. By symbiosis was formerly understood an intimate relation which is, physiologically, beneficial to both participants, and the lichens were given as an example of a real symbiosis. But there is nothing analogous in the above fermentations. There is really no longer a definable idea attached to the word symbiosis; it would therefore be best to cease using the word.

of *Sacch. apiculatus*, but the cells are far larger, and, besides, the appearance is quite different. The first stages of new growth are as in the preceding saccharomycetes: at a point on the cell a wart makes its appearance; but instead of a regular budding a septum appears by means of which the new cell will cut itself off. The genus *Schizosaccharomyces* has no budding whatever; but inside the cell this septum is formed. Thus *Sacch. Ludwigii* is in this respect related partly, and in fact most nearly, to the real saccharomycetes, and partly to the schizosaccharomycetes on account of the septum formation. Under certain conditions it forms a typical mycelium, especially in old cultures (p. 199, Fig. 77).

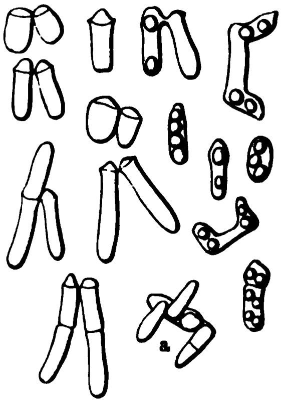


FIG. 103.—*Schizosaccharomyces Pombe*, Lindner. Vegetative and spore bearing cells. *a*, Germinating spores. $\frac{200}{1}$. (After Lindner.)

Hansen's researches on spore germination (Figs. 84 and 85) have been already alluded to. In this respect the species is also distinguished from the other saccharomycetes and might well be classified as a separate genus. During germination a promycelium is formed and the yeast cells are developed from the latter. Coalescence of the young spores usually takes place in the early stages of germination.

This species forms spores very easily on gypsum blocks gelatine, and also in various nutrient liquids, *e.g.*, in a per cent. saccharose solution. The spores are round, and to 4 μ in diameter. According to Nielsen the cardinal points of spore formation are the following :—

At 34° no spores are formed.
„ 32° to 32½° C. the first indications are seen after 19 to 21 hours.
„ 30° C. „ „ „ „ „ 18 to 19 „
„ 6¼° to 7½° C. „ „ „ „ „ 13 to 14 days.
„ 2½° to 3° C. no spores are formed.

Sacch. Ludwigii, according to Hansen, ferments dextrose and saccharose but not maltose. In dextrose-yeast-water it can produce up to 10 vol. per cent. of alcohol, but in beer wort only 1·2 vol. per cent. In a saccharose solution it sometimes dies off rather quickly, a behaviour contrary to that of most of the other *Saccharomyces* species.

2. *Genus : Schizosaccharomyces, P. Lindner.*

Vegetative increase takes place by fission and not by budding. This fission occurs through a septum being formed nearly in the middle of the cell; the septum splits and the cell divides into two cells often hanging together as if by a hinge.

Schizosaccharomyces Pombe, P. Lindner (Fig. 103), was found by Saare in Pombe (negro millet beer) from Africa. The two ends of the cells are often different; one is rounded, the other is encircled by a well-defined circular ridge which encloses the newly formed conical membrane. In exhausted culture solution the cells become shorter.

Spores are formed to the number of 1 to 4 in each ascus; they form more easily in culture liquids than on gypsum blocks. Germination takes place by means of a germinating tube. A film is not formed. The optimum temperature for the growth is 30° to 36° C. This species forms much alcohol, the large amount of which has no deleterious influence on the growth. It is a yeast, and ferments maltose, dextrose, sacchar dextrin: it contains invertin. This fungus is advantage in South American distilleries, as it stand the warm climate.

Schizosaccharomyces octosporus, Beijerinck 104 and 105), was found by Beijerinck on curra Greece, and by Schiönning on Italian raisins. Som cells are cylindrical, some oval, and in fresh wort they are 4·5 to 6 μ broad and 7 to 13 μ long.

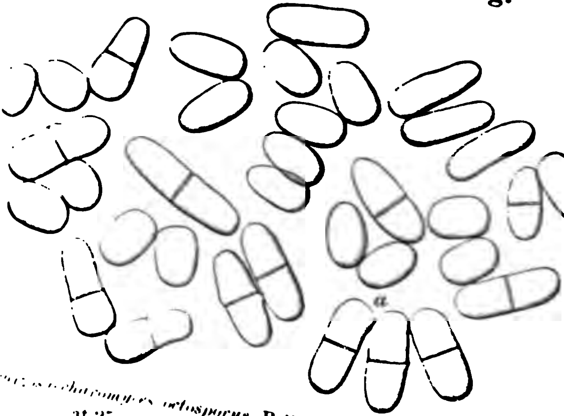


Fig. 104.—*Schizosaccharomyces octosporus*, Beijerinck. A young growth in wort at 25° C. 1000/1. (After Schiönning.)

The remarkable ascus formation in this species (Fig. 86) observed by Schiönning is described on page 211. The number of spores is eight as a rule, but somewhat frequently only four are found; the number may also vary from two to seven. The shape of the ascus varies; so also does its size; the breadth is 6 to 10·5 μ, and length 14 to 20·5 μ. The size of the spores is 3 to 5 μ. The latter are stained blue with a solution of iodine in potassium iodide (Lindner). They are formed copiously on solid nutrient substrata, more scantily on gypsum blocks and in wort cultures.

ording to Seiter they are formed on gypsum blocks at in six to seven hours. This species develops no film on rt, but at room temperature a weak yeast ring is formed a month.

It ferments maltose and dextrose, but not saccharose. hiönning states that this fungus forms in beer wort bout 14 per cent. Ball.) 4·6 vol. per cent. of alcohol in ree weeks at 25° C., and 6·56 vol. per cent. of alcohol in ive months.

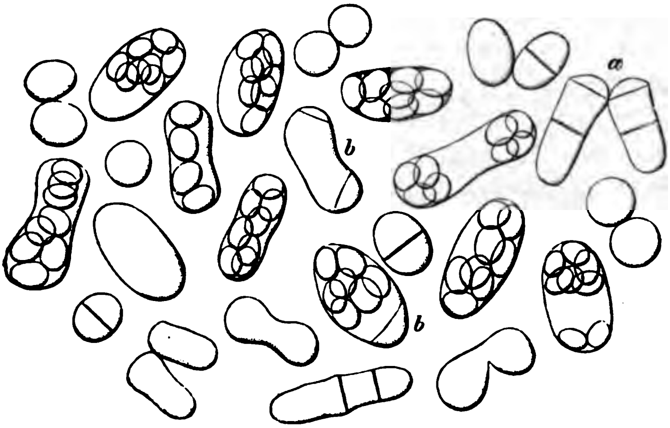


FIG. 105.—*Schizosaccharomyces octosporus*, Beijerinck. A young growth on wort gelatine; some cells contain spores. $\frac{1000}{1}$. (After Schiönning.)

Schizosaccharomyces mellacei, Jörgensen, was isolated by Greg from Jamaica rum. The spores of this species are stained blue by iodine in potassium iodide (Holm). Altogether Greg is said to have found eight species of *Schizosaccharomyces* in the mashes in rum manufacture.

Order II.—Perisporaceæ.

The asci are enclosed in the so-called perithecium; the latter is an envelope more or less spherical, completely closed, and consisting of one or several layers of cells (Fig. 106 c).

The conditions governing the formation of these perithecia have been studied by Hansen and Klebs. Hansen made most of his experiments with *Anixiopsis stercoraria*, Hansen, a fungus which grows in the open air on manure, and which also thrives in wort and on wort gelatine. He found that in cultures on wort gelatine and wort agar gelatine the limits for mycelium formation lie in the neighbourhood of 36° C. and 2° C. At 25° C. a vigorous formation of perithecia takes place, but it is over long before 35° C. is reached, and its minimum limit is near 8° C. It is thus seen that the temperature maximum for the development of the mycelium and gemmæ lies remarkably higher than for the development of perithecia, and also that the development of mycelia and gemmæ has a lower minimum temperature than the development of perithecia. Therefore, growths may be prepared, according to wish, with or without perithecia. This behaviour is exactly the same as he observed in the saccharomycetes as described already (p. 210), namely, that the budding of yeast still proceeds at temperatures which, on the one hand are higher, on the other are lower, than those at which spore formation can take place. He puts this down as a general rule for most fungi.

Klebs found that perithecium formation can always be brought about with absolute certainty in *Aspergillus repens*, de Bary, on new bread between 25° and 32° C. Below 25° C. the result becomes uncertain; between 12° and 22° C. no fructification is in general observed. On cultivating this species on 80 per cent. dextrin he further found that only conidiophores are developed if the cultivation takes place at 16°, but that if a temperature of 28° C. is employed, the growth consists almost entirely of perithecia. At 15° C. formation of perithecia also takes place, but only if the formation of conidia is in some way restricted.

Vegetative increase occurs in many of the allied forms by means of conidia. Several species form sclerotia. This term is applied to certain hard tubercular bodies which have a rind, and are often dark coloured. They consist of a thick web of mycelium threads, and serve for storing reserve food stuffs. After a period of rest, the length of which varies, they germinate into fruit carriers or fruit bodies.

The Perisporaceæ are, with the following order, the Sphæriaceæ, divisions of the Pyrenomycetes.

Several *Aspergillus* and *Penicillium* species cause much damage in breweries by attacking the barley and malt, particularly the broken grains. J. Rauscher has carried out comparative experiments with wort from mouldy and non-mouldy malt and Lott later obtained the same results, *viz.*: (1) that mouldy malt gives less extract than normal malt; (2) the ratio between sugar and non-sugar diminishes; (3) the quantity of fermentable material decreases so that beer prepared from mouldy malt contains less alcohol; (4) the quantity of acid in the wort increases. On the other hand he found, in contradiction to previous observers, that the malt attacked by the mould does not cause a mouldy smell or taste in the wort nor in the beer prepared with it. Prior mentions that beer can of itself assume a mouldy taste, if it is merely lying in a mouldy cellar.

In laboratories these fungi may be kept, either in the dry state in filter paper, or in a 10 per cent. saccharose solution. In both cases they live for a great number of years. In the case of *Aspergillus glaucus*, Hansen found that this species kept alive in a paper preparation for more than sixteen years, and *Anixiopsis stercoraria* lived, under the same conditions, for more than twenty-two years.

[illegible] Moulds (Aspergilleæ).

On the conidiophores are flask-shaped bodies, sterigmata or basidia, from which the conidia are formed by abstriction (Figs. 106 1, 2, 107 and 108 A).

1. *Genus : Aspergillus, Micheli.*

The end of the conidiophore is inflated into a head. On this head the numerous small flask-shaped sterigmata are found from which the conidia are either directly detached, or the former all bear several smaller sterigmata from which the conidia are developed. Those species, in which the latter form of development takes place, are often classified in a genus by themselves: *Sterigmatocystis*, van Tieghem. Species are found, however, which exhibit both forms.

Ascus fructification (Figs. 106 5, 6) is known only in a few species which were formerly reckoned in the genus *Eurotium*, and we really ought therefore to class only these species with the ascomycetes till something further is known, while the others would be placed among the *Fungi imperfecti*. It was first demonstrated by de Bary that *Aspergillus* is the conidial stage of *Eurotium*. In some species a formation of sclerotia takes place without any appearance of asci, and in single forms gemma formation is said to appear under certain conditions of culture. Contrary to the following genus, *Penicillium*, in which growth goes on at very low temperatures, *e.g.*, near 0°, the aspergillæ favour higher temperatures.

Aspergillus glaucus, de Bary (Figs. 106 6-8), is a general designation for several species among which *A. repens*, de Bary (Fig. 107 1-5), may be mentioned specially. The growth forms on the substratum a covering at first bluish-green and later brownish. The conidia are 6 to 15 μ in diameter, spherical or somewhat ellipsoid and slightly warty. The perithecia, which are in the form of little yellow balls, appear in large quantity when the cultures are set away. (On the effect of temperature on their formation, see p. 272.) According to de Bary they are formed in the following manner: From the mycelium, side branches are developed which, after having stopped growing, become spiral shaped at the point (Fig. 106 3, 4). De Bary calls this screw or spiral the ascogonium. Side branches develop at the base of the latter and grow up; one reaches the end of the spiral first and the two grow together (Fig. 106 4) so that the plasma masses unite and fructification takes place by this means. The side branches all ramify and become divided by septa. The result of this is that finally a cell layer is formed round the ascogonium (Fig. 106 5); the former becomes yellow and forms the wall of the perithecium. Small branches grow out on the inside and also ramify so much that the whole space between the wall and the ascogonium is filled with a delicate texture (Fig. 106 5). The spirals of the ascogonium are thus separated, these having meanwhile been divided up by septa, and buds now grow at various parts of them. The latter branch and form asci at their ends (Fig. 106 6, 7), in which are produced 8 colourless spores about 8 to 10 μ in diameter, lens-shaped and provided with a grooved rim (Fig. 106 8).

Duclaux states that this fungus forms diastase which changes starch into dextrin and maltose.

It is extremely widespread in nature on dead plants and animal matter.

In maltings it occurs, as already stated, mostly on damaged grains. J. Behrens mentions that these and related forms can be found on hops, which then have a brown colour; it probably attacks the constituents of hops, transforming the salts of organic acids into carbonates.

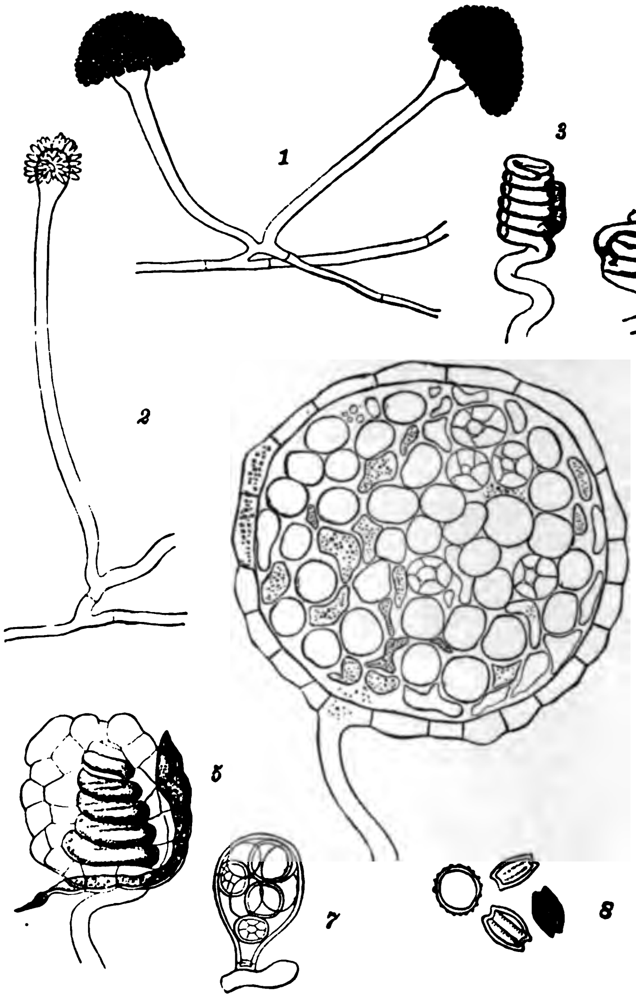


FIG. 106.—*Aspergillus repens*, de Bary (1-5), and *A. glaucus*, de Bary (6-8). 1. Conidiophores with spore chains. 2. Another without the spore chains, in order to show the sterigmata. 3, 4. Process of fructification. 5. Longitudinal section of a young perithecium, the spiral ascogonium in the middle; the outer shaded part represents a piece of the external yellow covering. 6. Longitudinal section of an almost ripe perithecium; the asci, some containing spores, can be easily recognised. 7. A ripe ascus. 8. Ascospores seen from different sides. (After de Bary.)

Aspergillus oryzæ, Ahlburg.—The growth is at first yellow or yellowish-green and later brown. The conidia are either smooth or slightly warted, and are 6 to 7 μ in diameter. Perithecia have not yet been found; under certain unknown conditions sclerotia are formed (Schiönning and the author). The optimum temperature for the growth lies above 30° C. It forms both maltase and diastase.

The species plays an important part in East Asia, where it is employed on account of its powerful diastatic action in the manufacture of saké. Saké, or rice beer, has been the national drink of the Japanese for thousands of years. Its preparation is carried out in the four following stages: (1) Preparation of "koji"; (2) preparation of "moto"; (3) the true fermentation; and (4) pressing and clearing. The whole process lasts from November till February. It is begun by the above fungus transforming the rice starch into sugar by its diastatic action; the sugar is then fermented by one or more alcoholic yeasts either taken from the air or accidentally present in the material. It is thus an impure fermentation. "Koji" consists of rice grains which are grown over and penetrated by the mycelium of the fungus. It is prepared by sowing the conidia of the fungus, a yellow-green powder, "tane-koji," on steamed rice, the former then being allowed to germinate at 20° to 25° C. One vol. of conidia is said to turn about 40,000 vol. of rice into koji. The process is completed in the course of a few days. "Moto" is made by mixing steamed rice with water and koji to form a thick soup. After some days the whole mass begins to liquefy, the diastase of the fungus turning the starch into a solution of sugar. During this process the temperature is only a little above 0°. Fermentation then begins of itself, whereupon the temperature is raised to about 20° C., and later to 30°-35° C. After fourteen days the "moto" is ready; it is a liquid containing sugar, alcohol and lactic acid and particularly yeast cells which are used in the true saké fermentation. For the latter a large quantity of steamed rice, koji, moto and water is mixed up, and the whole is stirred up into a soup, placed in a fermenting vat and left to itself. The process finishes in two weeks. The fermented liquid is then pressed out. In the year 1888-89, 7·2 million hectolitres (4·4 million barrels) of saké were manufactured in Japan; from this the extensive use of the fungus may be seen.

Aspergillus fumigatus, Fresenius, has conidia which are at first bluish-green and later brown. According to Cohn it is the cause of germinating barley, which has not been properly turned, becoming warm. This species causes diseases in the air passages of mammals and birds.

Aspergillus niger, van Tieghem, belongs to the sub-genus

Sterigmatocystis, its sterigmata being branched. The colour of the growth is black. The conidia measure 3·5 to 4·5 μ in diameter, and are furnished with small warts. It forms spherical or cylindrical brownish-yellow or reddish-brown sclerotia, which are 0·5 to 1·5 mm. in diameter.

The fungus contains diastase, maltase, invertase and emulsin, and, according to van Tieghem, breaks up tannin into gallic acid and glucose. Wehmer states that it is one of the most active producers of oxalic acid, turning half of the sugar exposed to it into oxalic acid. It is not always capable of doing this, however; perhaps in this there are two similar species concerned. It belongs, like the preceding one, to the pathogenic species.

2. *Genus: Penicillium, Link.*

The conidiophore has septa and has short branches near the top. On the latter, as well as on the main axis, are formed flask-shaped sterigmata, on which are chains of conidia (Fig. 108 A). Sometimes the conidiophores are fasciculated; this form of development was formerly classed in a separate genus, which was called *Coremium*. Ascus fructification has been observed only in a very few species; it was found by Brefeld in *P. glaucum* and by Zukal in *P. luteum*, and is a somewhat rare occurrence in these two species. But it is said by Ray to occur frequently in *P. sacchari*. The conditions of its formation are not known with exactness, and it is thus always a matter of uncertainty whether this fructification will be obtained.

Wortmann found living *Penicillium* conidia in wines which were many years old and contained a high percentage of alcohol; their resisting power thus seems under these circumstances to be very great.

Penicillium glaucum, Link (Figs. 107 and 108) (*P. crustaceum*), is a collective species under which are grouped a rge number of forms which differ only slightly, and which n scarcely be distinguished from one another by means of e characteristics at present at our disposal. The colour of e growth, initially white, then bluish-green, later grayish-reen, and finally often grayish-brown, is common to all. The conidia are spherical, and 2·5 to 4 μ in diameter.

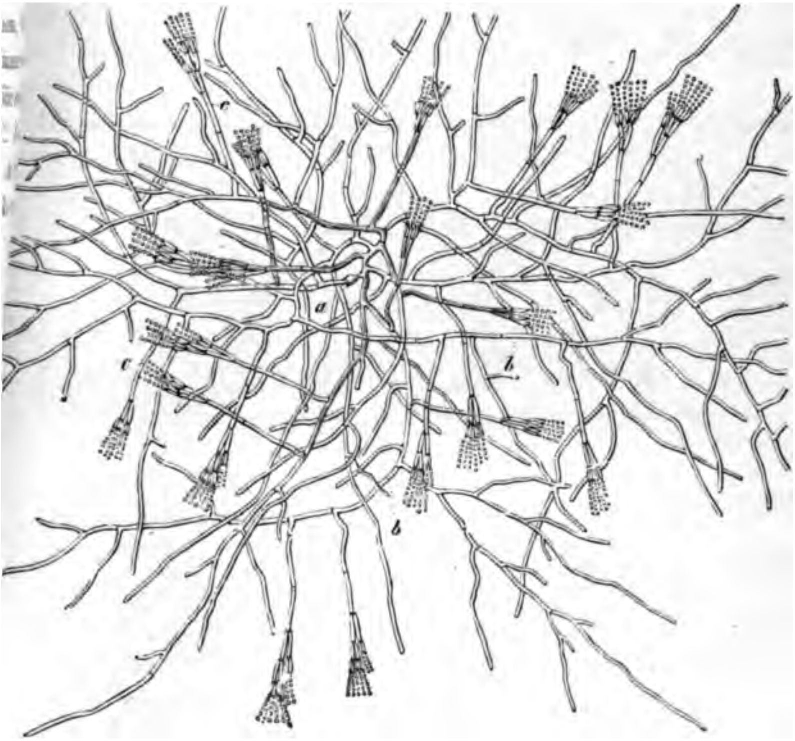


FIG. 107.—*Penicillium glaucum*, Link. *a*, The conidium originally seeded. *b*, Mycelium. *c*, Conidiophores. $\frac{120}{1}$. (After Brefeld.)

The ascus fructification discovered by Brefeld proceeds, according to him, in the following way : A yellow or brown sclerotium is first formed, 1 to 1·5 mm. in diameter ; this is produced by a screw-shaped ascogonium developing on a mycelium thread and being surrounded by a growth consisting of branched mycelium threads, which grow up partly from the base of the ascogonium and partly from the mycelium. This growth becomes gradually denser and harder; the ascogonium enlarges and its branches force themselves in all directions between the middle growth, consisting of thinner-walled cells. When such a sclerotium, fully ripe, is placed on damp filter paper, the ascogenous threads separate and push out thick side branches, the links of which finally change into asci. At the same time thin threads develop from the ascogenous hyphæ which penetrate into the sterile growth and dissolve it up. The foodstuffs thus obtained are taken by the fine threads to the ascogenous hyphæ. Finally, the dissolving process progresses so far that only the peripheral rind remains, while the inside appears filled up with spore masses. Perithecium formation takes place, according to Brefeld, only by abundant nourishment on bread. The ascospores, eight in each ascus, are yellowish, elliptical, 5 to 9 μ long and 4 to 7 μ broad.

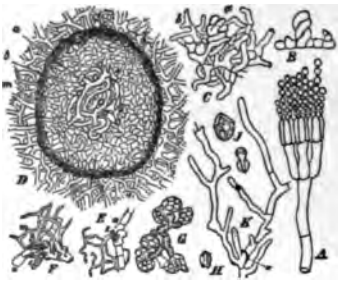


FIG. 108.—*Penicillium glaucum*, Link. A, Conidiophore. B, Sexual organs. C, Beginning of sclerotium (*a*, ascogenous hyphæ; *b*, sterile threads). D, Very young sclerotium in section.

The cardinal temperatures for the growth vary, according to Thiele, with the substratum. Thus the fungus is said to grow at 35° to 36° C. if glycerine and sodium formate present, whereas 4 per cent. of grape sugar impedes wth at a temperature above 31° C. The temperature imum is, on the contrary, unchanged by the substances ned.

Pasteur states that the conidia are killed if exposed for f an hour to a temperature of 127° to 132° C.; at 119° 121° C. they retain life. According to Lesage they are lled somewhat quickly by alcohol vapour, the times being x days, nearly one day, and two hours when subjected the vapour from 22·5 per cent., 45 per cent., and 90 er cent. solutions of alcohol respectively.

P. glaucum contains diastase and maltase (Bourquelot), lso a ferment which inverts cane sugar, and emulsin (Gérard). The diastatic action is, however, weakened if the content of the substratum in sugar increases; in a 10 to 15 per cent. cane sugar solution this fungus forms no diastase (Katz). It, further, breaks up tannin into gallic acid and glucose (v. Tieghem) and mandelic acid into its two optically active isomers, using the levo-acid in building up the cells (Lewkowitsch); this is said by Pfeffer, however, to be dependent for the most part on external influences. Under certain conditions it is said to be capable of forming mannite as the product of decomposition (Muntz). Rotten grapes with *Penicillium* growth may therefore produce a sick wine containing mannite. Calcium oxalate is deposited in the perithecia.

Mention of its occurrence on barley and malt, and its injurious action on wort and beer is to be found on p. 273.

When it is present in grape must, fermentation is much delayed. Müller-Thurgau is of the opinion that the cause of this is the formation of injurious substances, for fermentation is also restrained when the fungus is removed from the liquor before the addition of the yeast. Miyoshi found that it forms a specific protoplasm poison, and J. Behrens has shown experimentally its poisonous action on yeast as regards both fermentation and fermentative power. By a stronger nutrition of the yeast, *e.g.*, by addition of peptone to the nutrient solution, he succeeded in stopping this injurious action. The fungus causes the mouldy taste in wine, as well as a cork or stopper taste, by growing through the cork of the bottle (Wortmann). Like *Aspergillus*, it attacks hops, colouring them brown.

P. glaucum is very widespread in nature, and the air is full of its conidia. It is, therefore, one of the most dangerous guests in the laboratory, all the more as it only requires a very small quantity of nutriment for the support of life; but it also requires a certain amount of moisture. With regard to temperature, too, it is not particular; it thrives well at about 0°. It is frequently met with on fruit, kernel as well as stone fruit, and it is said to be able to penetrate the uninjured surface of grapes. Wehmer found it in herring pickle, and isolated it by means of plate cultures on nutrient gelatine, containing 10 per cent. of common salt, on which substratum it grew easily.

It betrays its presence by its characteristic smell.

Order III.—Sphæriaceæ.

The fungi belonging to this order have, with one exception and contrary to the *Perisporaceæ*, an opening in the dark-coloured perithecium. Conidia fructification takes place. This order includes a very large number of parasitical species.

SPHÆRIEÆ.

Genus: Sphærella, Cés. and de Not.

The almost spherical perithecia are set in the epidermis or in the uppermost layers of the web of the host and end in a simple, uncommon, papilla-shaped opening of fine skin-like consistency. The asci are connected in bundles; the spores are two-celled and colourless (seldom coloured).

Sphærella Tulasnei, Janczewski (Fig. 109), forms dark-coloured perithecia, frequently pear-shaped (Fig. 109 7), 0·3 to 0·4 mm. long and 0·15 to 0·20 mm. in diameter. At its surface, chiefly at the neck, numerous mycelium threads are often found which develop conidiophores (Fig. 109 7). The perithecia contain asci with eight spores (Fig. 109 8, 9), of which the topmost is larger than the others; its size is 28 μ in length and 6·5 μ in diameter.

The conidial form is one of those fungi which were formerly called *Cladosporium herbarum*, Link (Fig. 109 1, 2, 3). This name has been used, like *Penicillium glaucum*, *Aspergillus glaucus* and others, to designate several fungi. It is now impossible to say which species Link meant. Common to all is the mycelium, at first clear like water, and later olive green or brown, which sends out conidiophores that abstrict brown conidia often many-celled. The form named *Cladosporium herbarum* by Janczewski corresponds with that usually so called (Fig. 109 1-4). He describes the conidia as oval, either undivided or 2 to 5 celled (Fig. 109 5). In the largest varieties they are 25 μ long and 10 μ broad; in the smaller forms they are only half as large. Their cell wall is olive brown, often warty, but smooth in the smaller variety. The young mycelium is uncoloured, and only assumes the olive brown colour gradually. For the rest, the appearance may be made out from Fig. 109.

Janczewski showed, by experiments with seedings on barley and rye, that this fungus is the conidial form of the above *Sphærella Tulasnei.* It grows here as a saprophyte, not as a parasite. As soon as the conidia were brought to germination on nutrient gelatine, a piece of the gelatine with germinating conidia was placed on a cut rye leaf which was kept in a very damp atmosphere. The mycelium then grew up; as soon as the point of the latter reached one of the stomata in the leaf, it forced its way through this into the leaf,

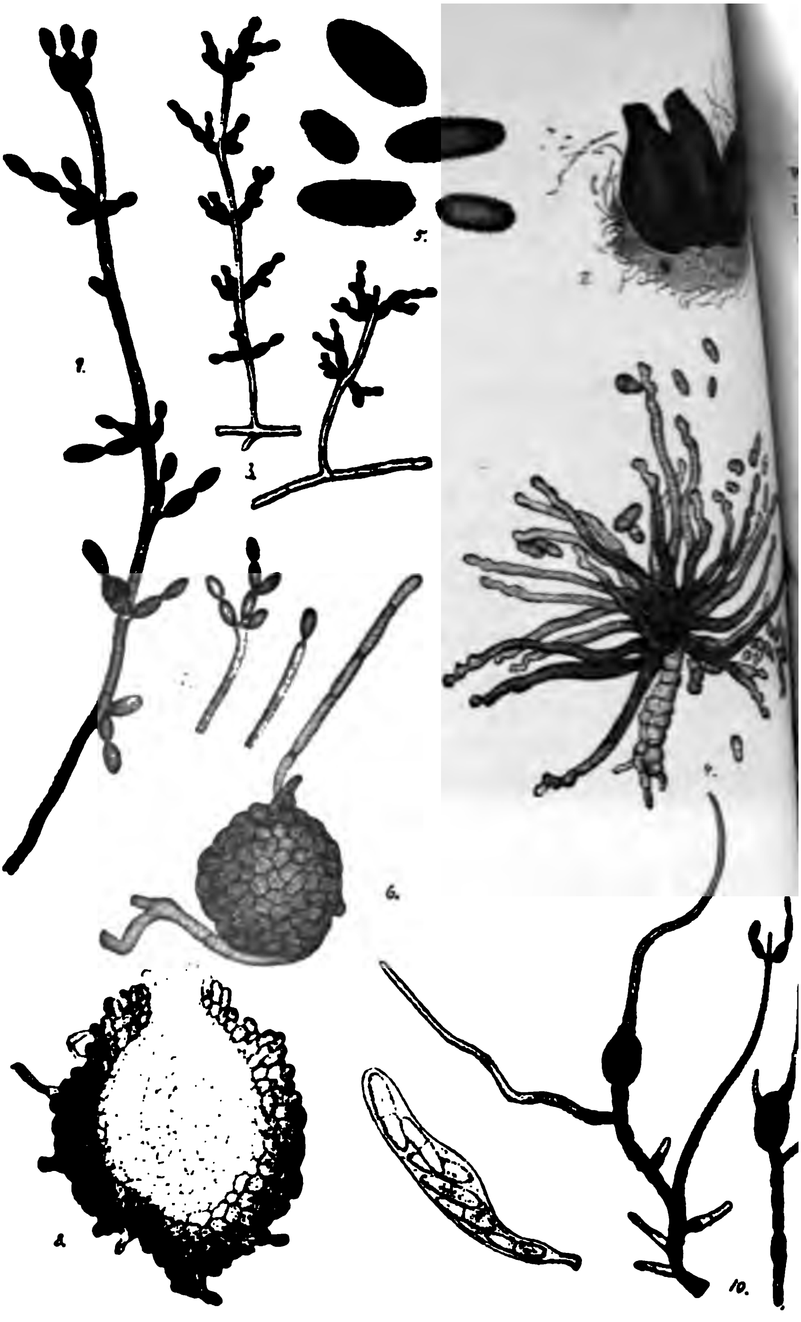


FIG. 109.—*Sphaerella Tulasnei*, Janczewski. 1, 2, 3. The conidial form *Cladosporium herbarum*, Link. $\frac{250}{1}$. 4. The same growing out through a stoma in the rye leaf sheath. $\frac{375}{1}$. 5. Conidia. $\frac{650}{1}$. 6. Sclerotium with a mycelium thread in a rye leaf sheath. $\frac{400}{1}$. 7. Perithecia with conidiophores. $\frac{6}{1}$. 8. Longitudinal section of perithecium containing two asci with endospores. $\frac{375}{1}$. 9. Ascus with ripe endospores. $\frac{400}{1}$. 10. Conidiophores developed from endospores. $\frac{250}{1}$. (After Janczewski.)

[illegible]re it continued to grow. When the seeding was done [illegible]inter (and only when done during the latter season), [illegible] mycelium often developed sclerotium-like bodies (Fig. 09 6), which were then transformed into perithecia. anczewski never found these bodies in nature on the eaves of any kind of corn. Placed on nutrient gelatine they were covered by mycelium threads extending radially and often in large quantity. After a few days this mycelium formed conidia. The transformation of sclerotia into perithecia takes place quickly. The ascospores germinate easily on nutrient gelatine, and, in a few days, develop mycelia with the conidial form *Cladosporium herbarum;* at the same time certain peculiar organs (Fig. 109 10) appear, the function of which is not known, and which are often found in the immediate neighbourhood of the conidiophores.

Cladosporium herbarum is very common on both dead and living plants. In breweries this fungus appears sometimes in large quantity on the walls of cellars; it is also found on malt, hops, etc. Like some other mould fungi it can give a cork taste to wine (Wortmann). It is not defined yet in how far it is identical with *Hormodendron cladosporioides* (Fres.) Sacc., yet both fungi are extremely like one another and the latter is probably often called *Cladosporium.* Janczewski says that he never succeeded in transforming *Cladosporium* into *Hormodendron*; but the mycelium developed on the above sclerotia sometimes exhibited a development of conidiophores similar to those of *Hormodendron.* Another similar fungus is *Fumago*, very thoroughly studied by Zopf, which is specially to be recommended to those who intend taking up this question.

Order IV.—Discomycetes.

The ascophores are open and have a very varied appearance; they may be either cup, disc, mussel or hat shaped.

The asci generally con
16, 32, 64, 128 and s
fructification is found i
formation.

CUP FU

1. *Genus* : S

The mycelium produces
ascophores grow under certa
fication takes place.

Sclerotinia Fuckeliana, de

This species is best known a
under the name *Botrytis cinere*
This fructification is usually deve
The conidiophores, 1 to 2 mm.
top like panicles; the ends of th
swellings which bear numerous fin
abstrict large conidia. When the
branches bearing them die, so that
take their place.

If the conidia are seeded out in an
tum, *e.g.*, in a thin layer of water, the
a very short germ tube; from the latter
shaped carriers small conidia (sperma
stricted, which, however, cannot be made
the other hand, if the large conidia ar
substratum, a typical mycelium is deve
forms either the usual large conidiophores
conditions, black sclerotia, a few millime
such a sclerotium is brought into a damp
mediately after it gets ripe, conidiophores
but, if it is kept at rest for a year at least, the
velops in the shape of long-stalked cup fruits

The spores of these asci may, like the large conidia, give either mycelia with large conidiophores or small conidiophores of which the conidia do not germinate.

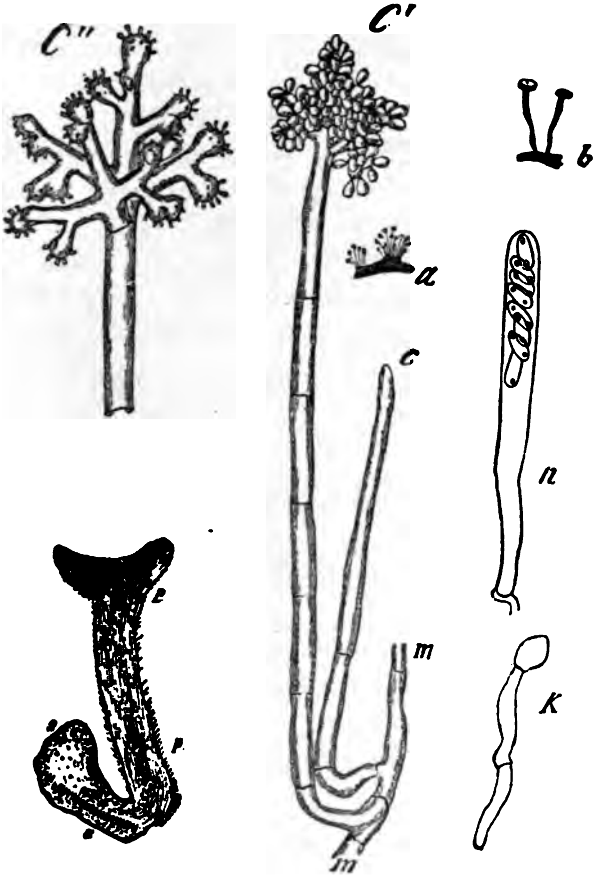


FIG. 110.—*Sclerotinia Fuckeliana*, de Bary. *a*, Sclerotium from which the *Botrytis* conidiophores have grown ; *b*, Sclerotium with two cup fruits ; C′, Conidiophore of the *Botrytis* form ; *m*, Mycelium. $\frac{200}{1}$. C″, End of a conidiophore with branches and sterigmata. $\frac{200}{1}$. *k*, Germinating conidium. $\frac{200}{1}$. *s*, Section of sclerotium with ascophore, *p*, (not much enlarged) ; *n*, An ascus with eight spores. $\frac{200}{1}$. (After de Bary.)

In *Botrytis cinerea*, P. Lindner frequently observed the phenomenon of inter-growth. According to his investigations, an irregular distribution of the plasma takes place in the older mycelia, some of the cells storing up large quantities of the contents, while others are completely emptied. The phenomenon of inter-growth is connected with this solely by the fact that the only cells which germinate inside the old mycelium are almost always rich in protoplasm. Fig. 111 represents a special case of this kind of germination, in which small conidia or spermatia are formed inside the cell. At the same time spermatia have also grown out laterally on the mycelium thread.

Oxalic acid is secreted in large quantity by the mycelia and sclerotia. Kissling showed that it forms a poison which kills living protoplasm. According to J. Behrens this poison is not an enzyme. *Botrytis* turns starch into sugar, and contains emulsin (Gérard).

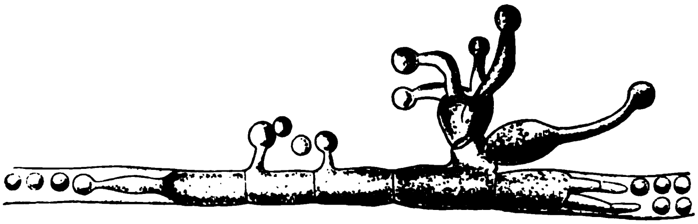


FIG. 111.—*Sclerotinia Fuckeliana*, de Bary. The *Botrytis* form. Phenomenon of inter-growth. Abstriction of small conidia (spermatia) inside a cell. (After P. Lindner.)

This species is very widespread in nature, and occurs on all putrefying plant matter. It occurs as a parasite on vines, both on the leaves and grapes.

Botrytis cinerea may sometimes play an important part in wine manufacture. According to Müller-Thurgau, under certain circumstances it induces the so-called "Edelfäule" in the grapes, which forms the basis for attaining the highest concentration of the grape juice, and especially for the appearance of peculiar bouquet substances, the so-called sherry bouquet, in wines; this occurs when it attacks quite ripe grapes, consumes the acid and, by rotting the skin, allows the water to evaporate, and thus increases in a very high degree the concentration and sugar content of the berry. Wines thus made from over-ripe (edelfaul) grapes ferment very slowly. The cause of this is the separation of the protoplasm poison mentioned above, which reacts injuriously on the yeast, as was shown by J. Behrens. This injurious action can be prevented to some extent by a more vigorous nourishment of the yeast.

Grape must, in which *Botrytis cinerea* has been cultivated, contains an oxydase which, according to some investigators, causes the disease of wine which is known as "maladie de la casse," and which consists in a precipitation of the colouring material. Culture solutions of *Botrytis* when mixed with equal quantities of sound wine cause the colouring matter to precipitate completely in about four hours. The disease may be prevented by heating up to 70° C., when the oxydase becomes inactive (Laborde).

The opinion obtained formerly that this fungus was responsible for the smoky flavour of wine, but this is not the case (Mach, Müller-Thurgau).

B.—IMPERFECT FUNGI (FUNGI IMPERFECTI).

A large number of the fungi which have been discovered one by one by mycologists cannot yet be classified in the system set up by them; these fungi are therefore grouped for the present under the above name. This applies also to the following organisms, which are of interest for the fermentation industry.

The Torula Species.

Originally the name *Torula* was given to hyphomycetes which had necklace-like, single or branched chains, of which the round or oval members were separable from one another. Later, however, the name included a number of different fungus species. Thus Turpin in 1838 calls *Saccharomyces cerevisiæ* Torula cerevisiæ, while the name *Torula* was afterwards given by Cohn to the necklace-like chains formed by the bacterium genus *Micrococcus*. *Torula* was understood by Pasteur to include yeast fungi with a very weak alcohol formation; he did not mention whether they formed spores or not. The species of this kind might, therefore, be true saccharomycetes; this is, however, not the case with Hansen's *Torula*. By *Torula* Hansen understands yeast cells which are similar to *Saccharomyces*, but do not form endospores nor develop typical mould growths. As regards the production of alcohol they may exhibit this in all degrees. According to the view held by the same investigator they will some time in the future probably be ranked with forms in the system widely separated from one another. As, however, we are ignorant on this point as yet, these organisms are for the time placed together in a group by themselves.

After Hansen had made clear the conditions for the formation of asporogenous varieties in the saccharomycetes, and since such asporogenous varieties can be formed in nature, it is comprehensible that several forms which appear under the name *Torula* possibly originate in saccharomycetes. There are *Torula* species which have all the physiological characteristics common to the saccharomycetes; this is true also as regards the morphological features, but of course with the exception of the property of greatest importance to the saccharomycetes, *viz.*, that of endospore formation.

Torula species are very widely distributed in nature. Hansen found them always present in the ground and in large quantity after winter in the hairy coats of bees and wasps as well as in their nests. The author also found them constantly when investigating a large number of these insects. Some of these fungi cause a disagreeable taste and smell in wort and, according to Wortmann, also in wines. The latter author found forms, in old bottled wines, which make must slimy. Rich. Meissner has in recent years isolated several species which cause wine to become viscous (see below).

Hansen has thoroughly investigated, among others, seven different species, which have, however, received no systematic name. These are as follows:—

Torula No. 1 (Fig. 112).—The cells are 1·5 to 4·5 μ in size. After long standing in wort, this fungus forms a scarcely appreciable amount of alcohol without any trace of frothing; it does not secrete any invertase.

Torula No. 2 (Fig. 113).—The cells are 3 to 8 μ in diameter. The protoplasm becomes granular in wort. Otherwise this *Torula* behaves essentially like No. 1.

Torula No. 3 is similar to No. 2. In wort it yields $\frac{7}{8}$ vol. per cent. of alcohol with a small but distinct production of froth, and does not form invertase.

Torula No. 4 (Fig. 114).—The cells are 2 to 6 μ in diameter. It inverts saccharose and, in wort, forms a little more than 1 vol. per cent. of alcohol with vigorous frothing.

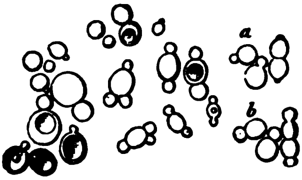


Fig. 112.—*Torula No.* 1. $\frac{1000}{1}$. (After Hansen.)

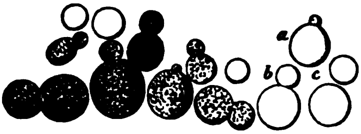


Fig. 113.—*Torula No.* 2. $\frac{1000}{1}$. (After Hansen.)

Torula No. 5.—This species soon forms a grey film over the whole surface of wort, yeast-water and lager beer; the film is only slight on a saccharose solution. The latter sugar is inverted by it; but in wort it produces no noteworthy fermentation and correspondingly only a trace of alcohol.

Torula No. 6 (Fig. 115) exhibits a distinct fermentation in wort and generates in it 1·3 vol. per cent. of alcohol. No fermentation takes place in a maltose solution. It inverts saccharose and in fifteen days at 25° C. generates 8·8 vol. per cent. of alcohol in a 15 per cent. dextrose solution.

Torula No. 7 (Figs. 116 and 117) was found in the soil under vines. It produces 1 vol. per cent. of alcohol in beer wort; on the contrary it excites no fermentation in solutions of saccharose, which it is unable to invert. In yeast water containing 15 per cent. of dextrose it formed 5·3 vol. per cent. of alcohol.

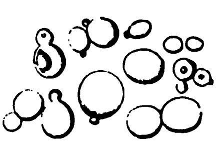


FIG. 114.—*Torula No. 4.* $\frac{1000}{1}$. (After Hansen.)

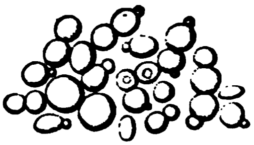


FIG. 115.—*Torula No. 6.* $\frac{1000}{1}$. (After Hansen.)

The last named species are perhaps active in wine manufacture, but hardly so in breweries and distilleries.

Rich. Meissner isolated eleven *Torula* species which all cause that disease called the ropiness ("Zähewerden") of wines; he has shown by experiments that must as well as wine becomes slimy, oily and thick when seeded with these. Most of these forms do not produce films, but only a yeast ring; must is decolourised by all of them. In the few species which form a film the latter was in some cases white, and in a single instance olive green. Only two of the eleven species referred to bring about alcoholic fermentation. Common to all is the need of oxygen, without which they cannot grow. If the nutrient liquid contains more than 5 vol. per cent. of alcohol, growth as a rule ceases, but at the same time the organisms are not killed. These slime yeasts check the fer-

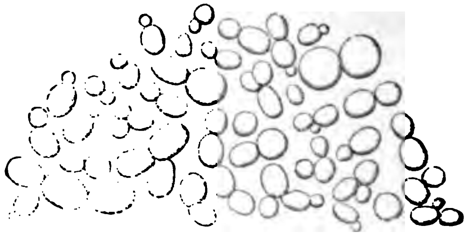


FIG. 116.—*Torula No. 7.* Sedimentary yeast. $\frac{1000}{1}$. (After Hansen.)

mentation, not of the strong yeasts, but only of feebly fermenting yeasts in the first few days of fermentation. The ropiness of wine occurs chiefly in those wines which are poor in tannin ; the disease can therefore be prevented by the addition of tannin, which latter checks the growth of the slime yeasts. The addition of a pure wine yeast is an especially favourable means for suppressing the slime yeasts so completely that the disease does not appear.

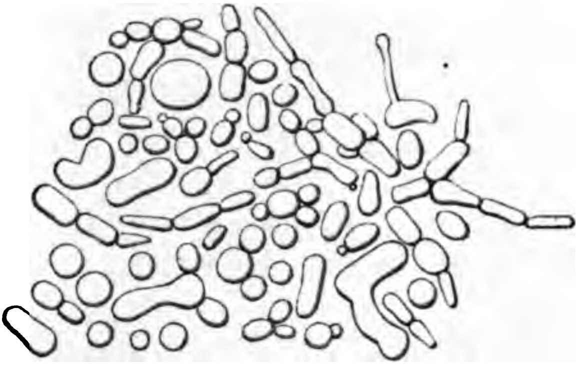


FIG. 117.—*Torula No.* 7. Film growth on a wort culture ten months old. $\frac{1000}{1}$. (After Hansen.)

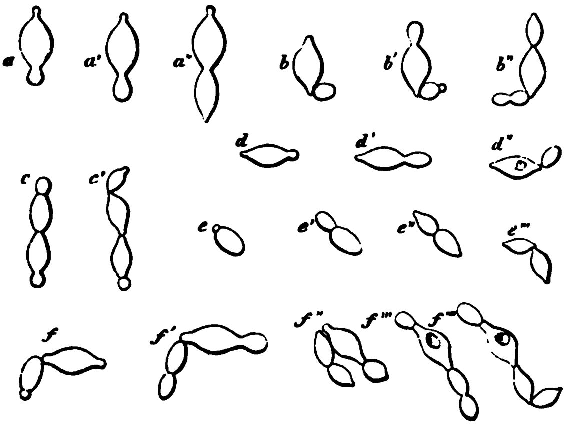
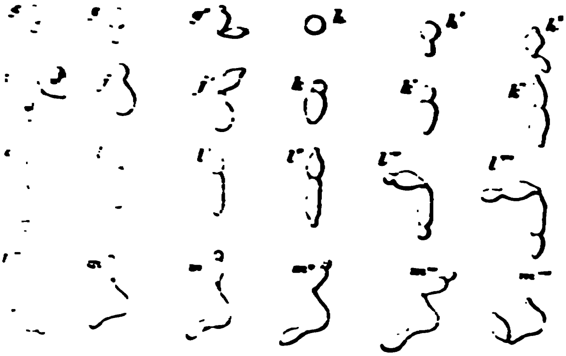


FIG. 118.—*Saccharomyces apiculatus*, Reess. (*a*) A cell which has begun to develop a bud ; *a'* and *a''* the same cell after the lapse of 1½ and 3¼ hours ; *b* another budding cell, *b'* after two hours, *b''* after three hours ; *c'* is ¾-hour older than *c* ; *d* was observed at 2½ P.M., *d'* at 3¼, *d''* at 3¾ ; *e* 10¾ o'clock, *e'* 12, *e''* 12¾, *e'''* 1 ; *f* 2½, *f* 3¼, *f'* 4, *f''* 5, *f'''* 5½ o'clock. About $\frac{250}{1}$. (After Hansen.)

Various rose-coloured budding fungi are also classed with the Torula species; these occur especially on starch-containing media and the so-called pink yeast ("Rosahefe") of German technology is classed along with them.

Saccharomyces Apiculatus, Reess.

A budding fungus which does not form endospores and which generally occurs in vineyards and orchards was given this name by Reess. It has been thoroughly studied by Hansen, and we owe the following to his investigations.



[illegible] Reess. Most of the cells are in the act of [illegible] show abnormal cells. The two cells *i* each [illegible] *g* was observed at 3¾ P.M., *g′* 5¼, *g″* 6½; *h* 10¼, *h′* [illegible] 12½, *k″* 1½, *k‴* 2¼; *l* 7, *l′* 8, *l″* 8 h. 5 m., *l‴* [illegible] 7, *m‴* 7¼, *m⁗* 7¾. About [illegible]. (After Hansen.)

The cells (Figs. 118 and 119), which are generally 6 to 8 μ long and 2 to 3 μ wide, are in some cases pointed at both ends like lemons, in others oval. The fungus forms both kinds of buds. In order to change into lemon-shaped cells the oval buds must grow through one or more buddings. The lemon form is produced more especially at the beginning of the budding and has then the preponderance; later the oval cells predominate.

It is a bottom yeast form which does not secrete invertase and in consequence cannot ferment cane sugar; it is also incapable of fermenting maltose. It therefore does not form more than 1 vol. per cent. of alcohol in wort; on the other hand it forms 4·3 vol. per cent. of alcohol in yeast water containing 10 per cent. of dextrose.

Sacch. apiculatus, like many other fungi, undergoes a remarkable variation. Thus Hansen found that of two growths investigated by him, one gave 3 and the other 4·3 vol. per cent. of alcohol. Amthor investigated two varieties, of which one furnished 3·25 and the other 4·56 vol. per cent. of alcohol, and Müller-Thurgau found that in seven cultivations in sterilised grape juice the alcohol production varied between 2·5 and 3·8 per cent. by weight. Will had two growths, one of which evolved a mouldy smell, the other an amyl ester-like bouquet. But whether such variations are permanent or not, or on what they depend, has not been investigated.

Its extraordinary power of multiplication is characteristic of *Sacch. apiculatus*. This and its competitive relations with *Sacch. cerevisiæ* have been mentioned on p. 230.

Hansen's investigations on the circulation of *Sacch. apiculatus* in nature are described on p. 246. This fungus is generally distributed in nature on fruits and also in soil. Müller-Thurgau found it in the latter to a depth of 20 to 30 cm., and Berlese to a depth of 36 cm.

According to Will it is found commonly in bottom fermentation breweries, but only in small amount. It is generally present in those Belgian breweries where beer is prepared by spontaneous fermentation.

According to the investigations of Müller-Thurgau and Wortmann it is especially detrimental in the manufacture of wine, as it has a retarding influence on the fermentation, but not, however, if the liquid contains 3 vol. per cent. of alcohol. It is most effective during the first stages of fermentation. *Sacch. apiculatus* possesses in a higher degree than true wine yeasts the power of decomposing and absorbing organic acids. Müller-Thurgau's experiments show that this property also asserts itself when it works simultaneously with true wine yeasts, as is the case in the progress of ordinary wine fermentations. Finally, by the formation of volatile acids and other products it is injurious to the bouquet and flavour of the wine. According to investigations by W. Seifert it formed the largest amount of volatile acid (0·064 per cent.) and volatile ester of six pure yeasts in the same grape must. The amount of ester expressed in cubic centimetres of $\frac{1}{10}$ normal alkali on 100 c.c. of wine corresponded to 10·8, while with the other yeast species it varied between 1·32 and 4·4. When it ferments grape must a cider-like taste and smell are exhibited. Although the increase of *Sacch. apiculatus* occurring on fruit and grapes is not prevented by the addition of pure yeasts to the must, yet, as experimental results show, its detrimental influence can be very considerably restrained by the addition of a quick-growing, vigorously-fermenting yeast (Müller-Thurgau). According to some French investigators *Sacch. apiculatus* yields a good cider with a strong bouquet; but, according to Müller-Thurgau, it has, on the contrary, a harmful influence on the cider fermentation.

Mycoderma Species.

These fungi are the so-called true film fungi which are distinguished by the rapid formation on nutrient liquids, particularly on beer and wine, of a covering film having air between the cells. The cells are usually short and sausage-shaped. They are strongly aërobic.

Mycoderma cerevisiæ, Desm. (*Sacch. mycoderma*).—Several species are included under this name. The species usually to be found in the Copenhagen breweries forms a dull, gray, wrinkled film on wort and beer. The cells contain from 1 to 3 refractive granules, which are of a fatty nature. This fungus does not induce fermentation, contains no invertase, and occurs in practically all lager beer, but does not succeed in growing so long as the bottles are well stoppered.

It is known with certainty that at least some of these forms do no harm in breweries under ordinary conditions; this holds good with regard to the species observed by Hansen, A. Petersen, Grönlund, Jörgensen and Prior. Bělohoubek and Kukla, on the contrary, mention a species which causes turbidity in beer. The same applies also to three forms observed by Lasché in America, which are said to cause a bad smell and taste in beer. They are said, moreover, to produce alcohol in wort. Lafar has described an allied species which forms acetic acid in beer.

These forms are easily obtained if beer or wine is allowed to remain at a temperature of 10° C. with a free supply of air.

Mycoderma vini, Desm. (Fig. 120), is very nearly related to, or is probably identical with, the above species; it forms the film of wines. This film can become over 1 cm. thick. The fungus acts, like the other species, as an oxidising agent on the alcohol in the wine, forming carbonic acid and water. It can also attack other constituents of the wine. By decomposing a part of the free acid it favours the growth of acetic acid bacteria, and consequently the production of a vinegar taint. Wortmann states that this fungus can also influence directly the flavour of a wine.

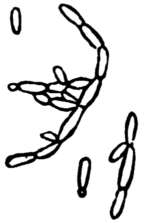


FIG. 120.—*Mycoderma vini*, Desm. About $\frac{620}{1}$. (After Wortmann.)

Forti mentions a *Mycoderma* species which has a detrimental influence on yeast in wine.

W. Seifert has made complete experiments with two related forms isolated from red wine, which he names *Mycoderma vini I.* and *II.*

The cells of **Mycoderma vini I.** are 3 to 10 μ long, and 2 to 4 μ broad. The films are smooth at first, later strongly wrinkled, coherent and grayish-white. The temperature limits for growth in wine with 8 vol. per cent. of added alcohol are: Maximum, 30° C.; optimum, 25° to 28° C.;

and minimum, 5° to 6° C. This species grows even in the presence of 12·2 vol. per cent. of alcohol, and vigorously attacks malic acid. In an artificial culture liquid (Pasteur's solution) containing malic acid and 4·8 vol. per cent. of alcohol, it formed 0·152 per cent. of glycerine in fourteen weeks; at the same time the whole of the alcohol had disappeared. In ordinary Austrian white wine it increased the amount of glycerine (0·68 per cent. to 0·82 per cent.), formed acetic acid (0·904 per cent.), and reduced the amount of alcohol (7·8 to 3·8 vol. per cent.) in twenty-six days.

Mycoderma vini II. differs from the above species in having temperature limits for its growth in wine with 8 per cent. of alcohol as follows: Maximum, 28° to 30° C.; optimum, 22° C.; and minimum, 1° to 2° C. This fungus attacks malic acid only to a small extent. In the culture solution referred to above, it only formed 0·016 per cent. of glycerine after fourteen weeks, and at the same time the amount of alcohol was only lowered from 4·8 to 4·1 vol. per cent.

No increase of glycerine was effected in white wine after twenty-six days, only 0·064 per cent. of acetic acid was formed, and the alcohol was only reduced from 7·8 to 6·8 vol. per cent.

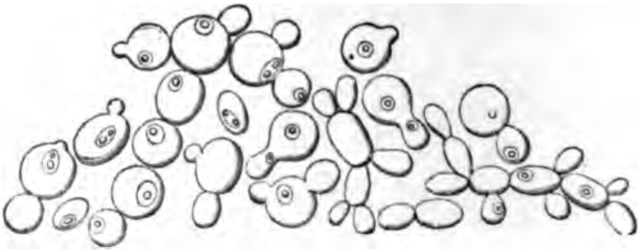


FIG. 121.—*Monilia candida* (Bonorden), Hansen. Sedimentary yeast. Vacuoles with refractive granules occur in some of the cells. $\frac{1000}{1}$. (After Hansen.)

Tartaric acid is practically not attacked by either species, and citric acid not at all. The glycerine and acetic acid formed are gradually used up again.

Monilia candida (Bonorden), Hansen.

This fungus (Figs. 121, 122 and 123) is generally found in nature on fresh cow dung and on fruit. The following investigations described are due to Hansen:—

In nutrient liquids containing sugar this fungus quickly develops a growth of *Saccharomyces*-like cells, in which vacuoles with one or two strongly refractive granules frequently occur (Fig. 121). When such a culture is allowed to remain some time the cells become elongated, and there results finally a complete mycelium, a mealy, white, tufted growth of mould which forms chains of yeast-cell conidia or divides into members like *Oidium* (Fig. 123, *d*). This growth also appears on solid culture media.

When young and vigorous cells of this species are seeded in a fermentable nutrient solution, *e.g.*, beer wort, a rapid and vigorous fermentation like a top fermentation is effected; even while the bubbles are rising a film forms on their surface. When the frothing has finished, the film gradually extends over the whole surface; during this process the large film-covered air bubbles gradually burst, and often cause folding of the film. If old cells are seeded the film forms before there is any perceptible macroscopic sign of fermentation. In wort after sixteen days this species formed 1·1 vol. per cent. of alcohol; after nine and a half months, 6·5 vol. per cent.; and after twenty-six months, 6·7 vol. per cent. After this time the maximum had been reached and the cells were dead. It formed 5·5 vol. per cent. of alcohol in 15 per cent. dextrose-yeast-water in fourteen days at 25° C.; in a 10 per cent. saccharose solution in twenty days, 0·7 vol. per cent. of alcohol; in six months, 3 vol. per cent.; and in twenty-seven months,

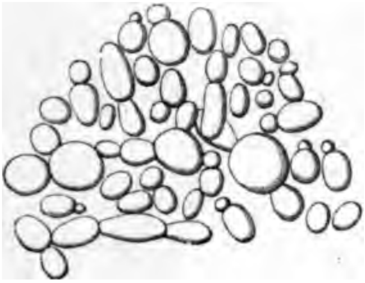


FIG. 122.—***Monilia candida*** (Bonorden), Hansen. Cells derived from a young film growth. (The shining granules appearing in Fig. 121 are not shown here.) $\frac{1000}{1}$. (After Hansen.)

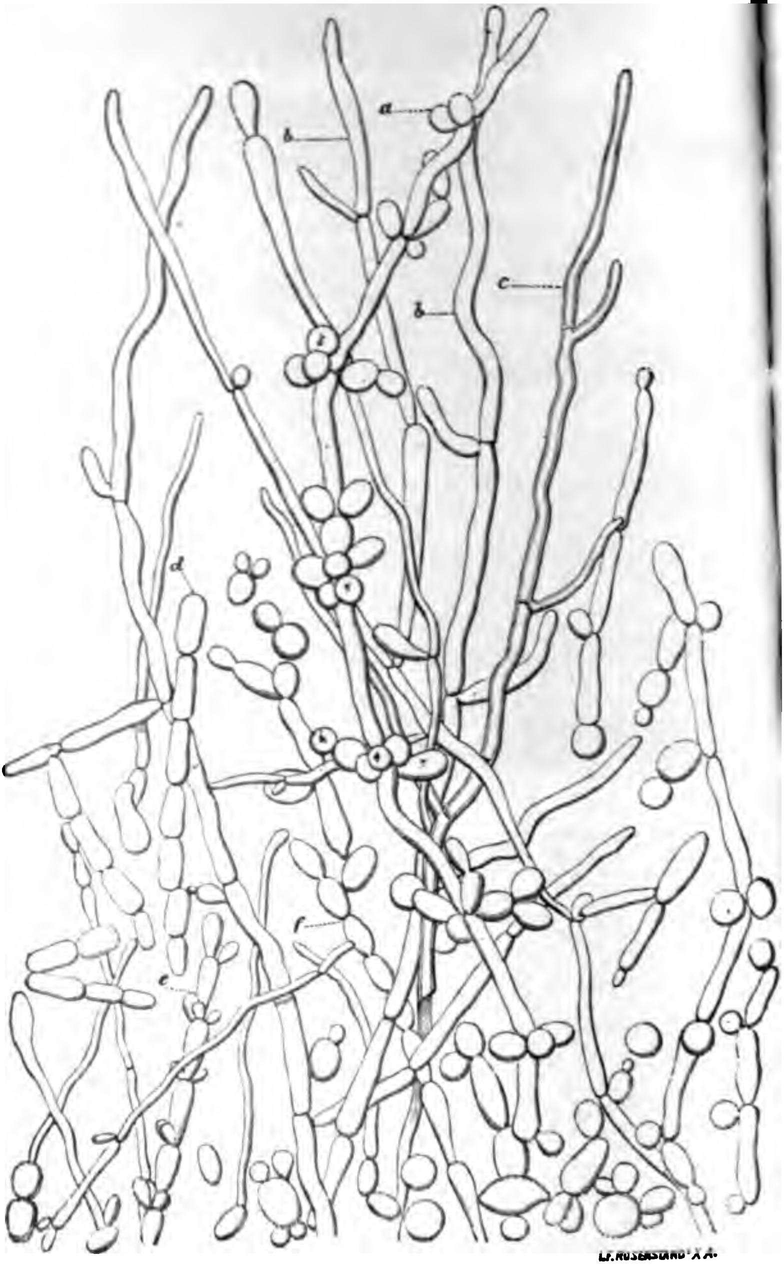


FIG. 123.—*Monilia candida* (Bonorden), Hansen. Mould growth from an old culture. *a*, Chains of more or less thread-shaped cells ; **at each node a whorl** of oval-shaped yeast cells often occurs. *b*, **The same form, but without oval** yeast cells. *c*, **Typical mycelium with septa.** *d*, *Oidium*-**like cells.** *e*, **Pear-shaped cells.** *f*, Lemon-shaped cells. $\frac{1000}{1}$. (After Hansen.)

4·9 vol. per cent. The cells were then still alive. The fermentation proceeded very slowly in these cases. The fungus withstands high temperatures, *e.g.*, it ferments vigorously at 40° C. In wort, the temperature limits are 42° to 43° C. and 6° to 4° C.

It is a remarkable circumstance that in the fermentation of the saccharose solution neither invertase nor invert sugar could be detected. Consequently the vigorous fermentation generated in the saccharose solution by *Monilia candida* must be quite unique, because saccharose was only known to be fermented after previous inversion. The invertase not being detectable by existing chemical methods, it follows that the fermentation must be regarded as a direct one. Nevertheless, Hansen indicated the possibility that the inversion takes place inside the cells and that the invert sugar produced is fermented as soon as it is formed. E. Fischer and P. Lindner by grinding the cells have recently discovered an invertase insoluble in water, *i.e.*, a ferment which is closely connected with the plasma of the cell. At the same time they found that this species contains maltase. According to Bau the fungus can ferment diastase dextrin.

Monilia javanica, Went and Prinsen Geerligs, occurs in "Raggi," which is applied in the manufacture of arrack in Java (see p. 260). On solutions containing sugar this species forms a film, which, however (and this distinguishes it from the previous species), disappears as soon as fermentation begins. It further differs from *M. candida* in that it inverts saccharose in the usual way, and the latter is then fermented; it also ferments dextrose, levulose, maltose and raffinose. When 5 per cent. of alcohol has been formed, growth and fermentation cease. The alcohol it produces has an unpleasant smell and taste.

A *Monilia* species has been described by Forti which has a detrimental influence on the yeast in wine.

Chalara mycoderma, Cienkowski.

Like *Monilia*, this fungus (Figs. 124 and 125) forms a film on liquids; it is composed of a branched mycelium which abstricts here and there globular or oval, but seldom pear-shaped conidia, 4 to 11 μ, most generally 4 to 6 μ in greatest diameter. They are formed by abstriction, in part from sterigmata and partly from the surface of the limb. This species thrives on wort and lager beer. It is found in the free state on grapes and on cow dung.

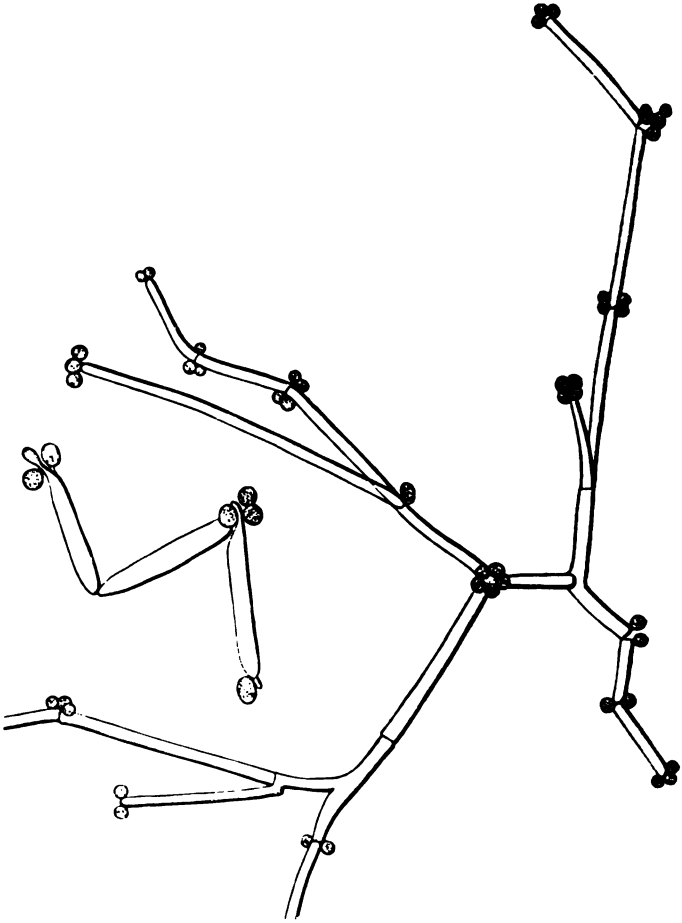


FIG. 124.—*Chalara mycoderma*, Cienkowski. Connected mycelium with conidia. $\frac{480}{1}$. The figure to the left shows separated mycelium branches and conidia. $\frac{780}{1}$. (After Cienkowski.)

Oidium lactis, Fresenius.

Oidium lactis, Fresenius (Figs. 126, 127 and 128), develops colourless, branched hyphæ, which form a white felt. The conidia develop by a division of the threads, and have, as a rule, a rectangular longitudinal section, but other forms are also to be observed; their length is most generally 10 to 30 μ, and breadth 3 to 5 μ. The temperature limits for the growth in wort are near 37½° C. and below ½° C.; for the film formation 36½° to 37½° C. and about 3° C.

An inter-growth similar to that observed by P. Lindner in *Botrytis cinerea* was found by the author and Schiönning in *Oidium lactis* (Fig. 128). When, for example, a young, vigorous mycelium is seeded in a thin layer of water, a more vigorous cell here and there grows into its feebler neighbouring cell and there forms conidia chains.

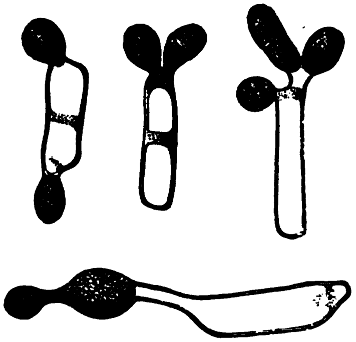


FIG. 125.—*Chalara mycoderma*, Cienkowski. Mycelium members, abstricting conidia. $\frac{1180}{1}$. (After Hansen.)

This fungus is found in general on milk which has been standing. According to Hansen's investigations it generates a trace of alcohol in wort and dextrose yeast water. According to Lang and Freudenreich it can generate 1 vol. per cent. of alcohol in milk-sugar and dextrose solutions.

In breweries it is to be found on malt, lager vessels, casks, piping, etc. It is also pressed yeast. Jörgensen states

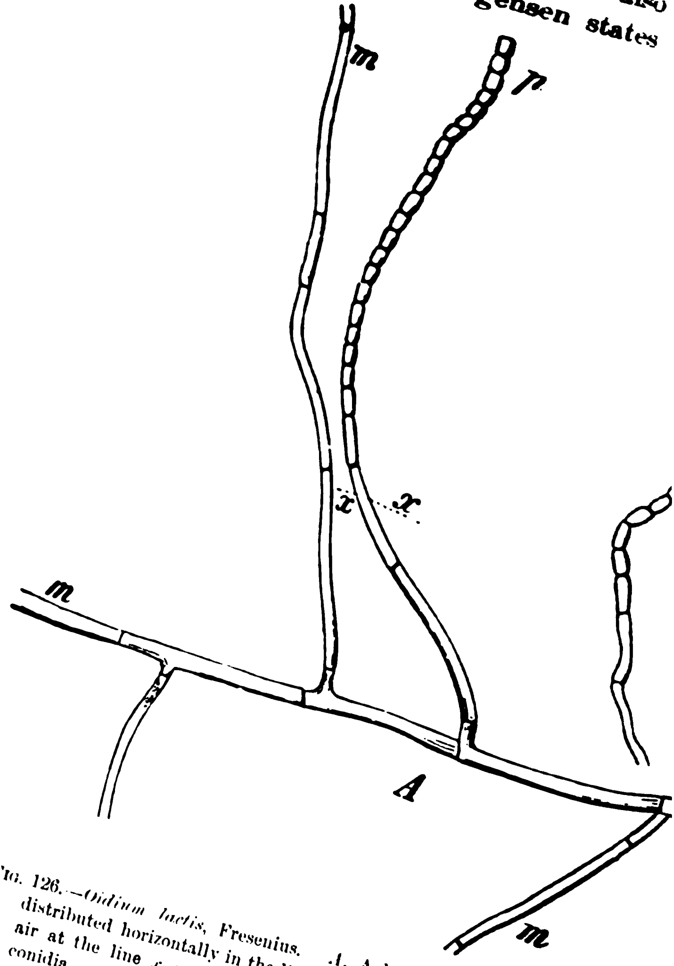


FIG. 126.—*Oidium lactis*, Fresenius. *A*, A branched mycelium thread *m-m*, distributed horizontally in the liquid medium; projecting obliquely into the air at the line *x-x*, a branch divided by septa into a chain of cylindrical conidia, *p*. *B*, Conidia chain at the commencement of the separation of its members from each other. About $\frac{390}{1}$. (After De Bary.)

large amount on top yeast when this is allowed to remain at rest after fermentation has ceased.

Oidium conidium formations have been observed by Brefeld in many mushroom fungi (*Agaricineæ*). Some botanists are therefore of the opinion that *Oidium lactis* is really a stage of development of such fungi. Proof of this has not yet been advanced.

Dematium pullulans, de Bary.

The fungus (Figs. 129, 130, 131 and 132) designated by this name has a branched, colourless mycelium on the surface of which, apparently without order, it frequently produces yeast cells or conidia by budding; the latter are mostly oval. These yeast cells are often situated on the threads on small humps which are sometimes discernible after the release of the yeast cells. They can then either produce more yeast cells by budding or develop germ threads, which grow into mycelia. Swollen parts are frequently found in the mycelium, which, after a certain time, become thick-walled and dark coloured, usually greenish-brown; the single cells developed by budding may also undergo transformation in this way. Gemmæ, which, among other ways, can be recognised by their contents (large oil and fat drops), are thus formed. *Dematium pullulans* does not induce fermentation.

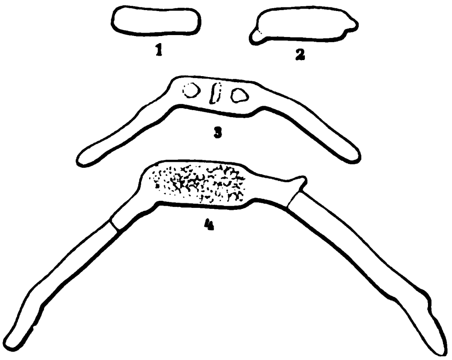


FIG. 127.—*Oidium lactis*, Fresenius. The germination of a conidium in wort in a Ranvier's chamber. 1 at 10½ A.M., 2 at 2 P.M., 3 at 4½ P.M., 4 at 7¾ P.M. $\frac{620}{1}$. (After Hansen.)

Fig. 130 represents an inter-growth similar to that which has been described under *Botrytis* and *Oidium*. Such endogenous conidia have been regarded and described as endospores. The author has shown, however, in conjunction with Schiönning, that in these cases it is a matter of inter-growth only. The phenomenon is brought about when a vigorous mycelium cell in the immediate neighbourhood of a feebler cell acts as a parasite on the

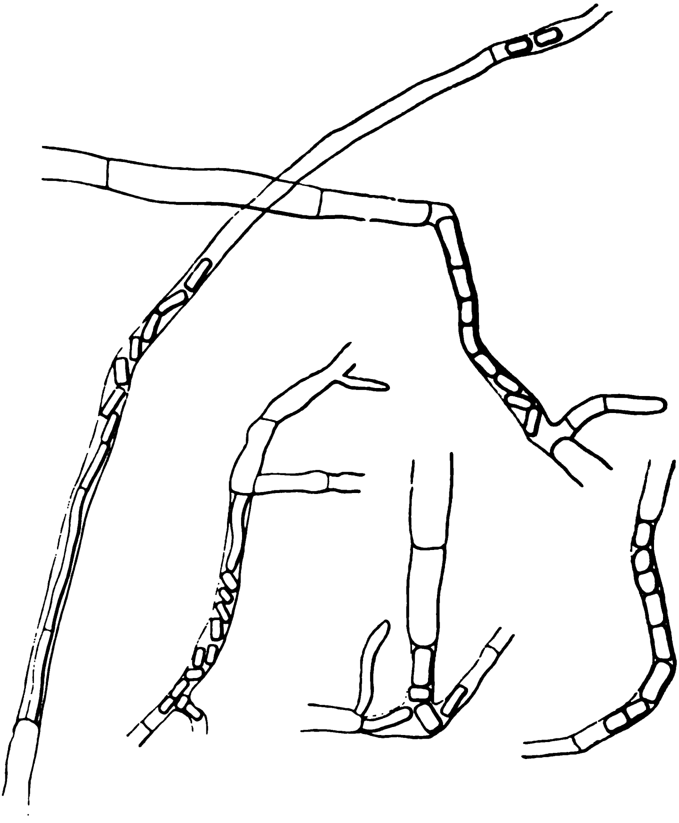


FIG. 128.—*Oidium lactis*, Fresenius. Phenomenon of inter-growth. $\frac{500}{1}$.
(After Klöcker and Schiönning.)

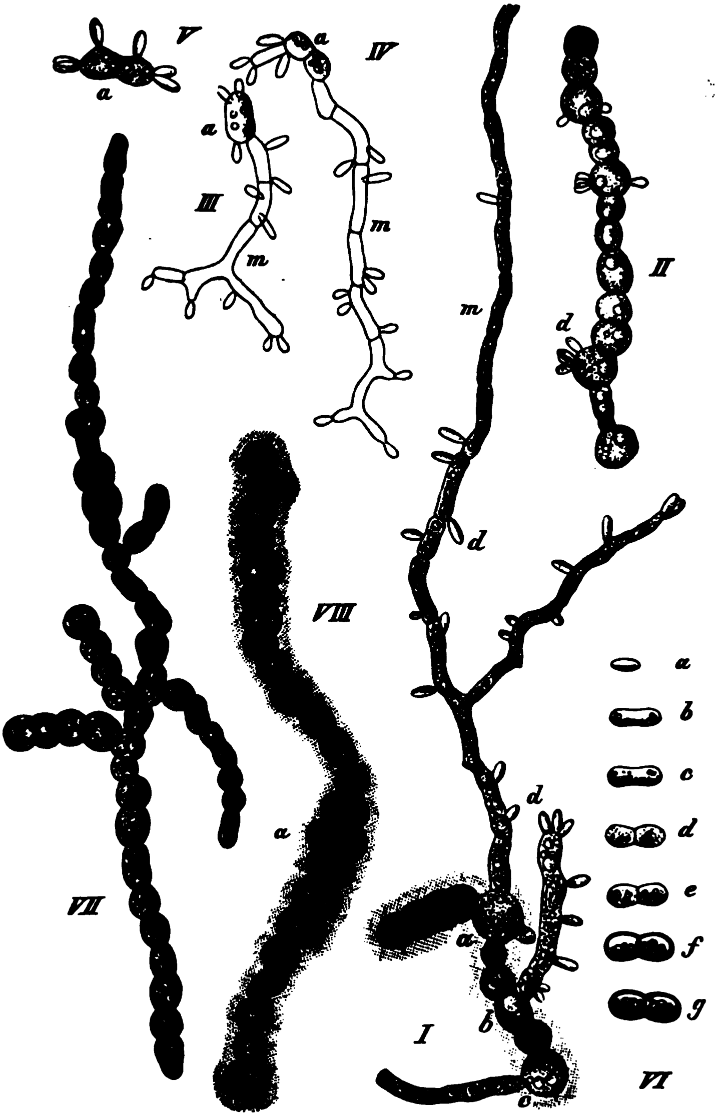


FIG. 129.—*Dematium pullulans*, de Bary. *I*, A chain of gemmæ; three members of this (*a*, *b*, *c*) have produced mycelium tubes (*m*) on which conidia (at *d*) are developed. *II*, A chain of gemmæ developing conidia directly (at *d*). *III*, A conidium *a*, which has grown a mycelium thread *m*, on which conidia are formed; it has also formed conidia directly. *IV*, Conidium divided into two cells under similar conditions. *V*, Conidium divided into two cells developing conidia. *VI*, *a-g*, Continuous development of one and the same gemma, in a very shallow water layer with free air supply, into a double-celled, thick-walled, brown gemma rich in fat. *VII* and *VIII*, Mycelia divided up into simple, short, swollen members, which have become thick-walled gemmæ, generally much browned and furnished with large oil drops. At *VIII a* some of the gemmæ are seen still further divided by septa, which lie in the same direction as the axis of the thread. $\frac{240}{1}$. (From Zopf's handbook.)

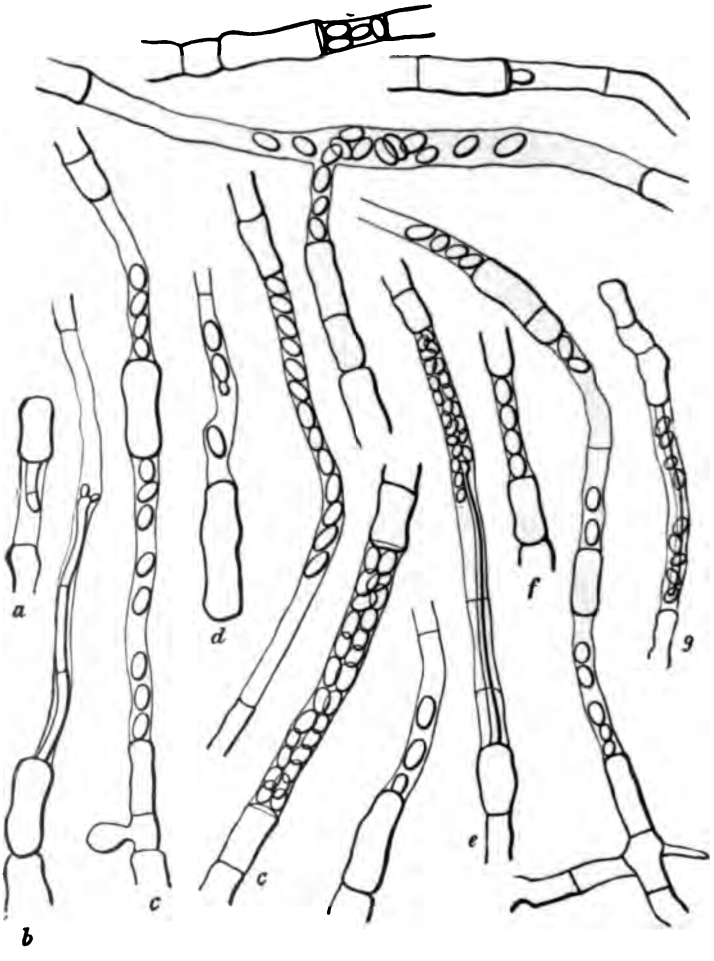
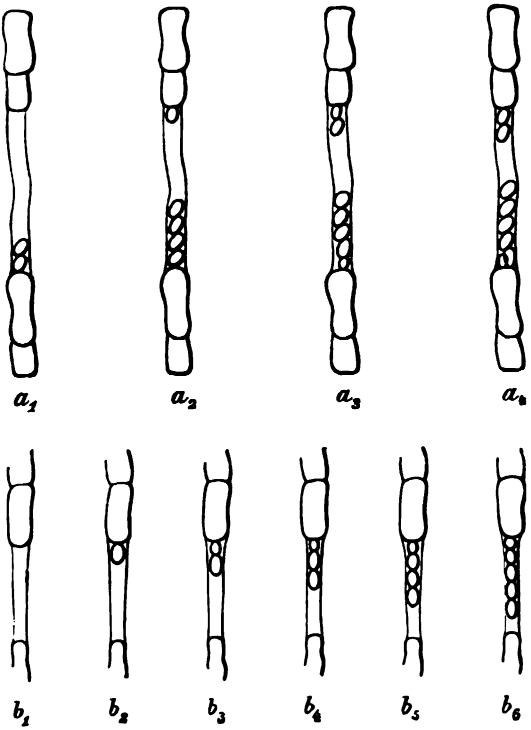


FIG. 130.—*Dematium pullulans*, de Bary. Phenomenon of inter-growth. *a* and *b* show intruded mycelium threads, in *b* the thread is forming conidia. *c*, Conidia are being abstricted on both sides. *d*, A conidium in the act of budding. *e*, A mycelium thread has grown through the septa of two cells into a cell filled with conidia. *f*, Cell with four conidia strongly resembling endospores. *g*, One of the cells has developed conidia, a mycelium thread has grown in from the other. The remaining figures show various examples of endogenous conidium formation. *e* was observed at about 20° C. in a water culture about one month old ; the remainder were observed in water cultures two days old at 20° and 25° C. $\frac{500}{1}$. (After Klöcker and Schiönning.)

er, and forms chains of yeast conidia at the end next and within the feebler cell; or, less frequently, the orous cell injects a longer or shorter mycelium thread g. 130 *a*, *b*, *e* and *g*). The yeast conidia formed in the erior of the cell may further increase here by budding



131.—*Dematium pullulans*, de Bary. Two development series of endogenous conidium formations directly observed in cover glass water cultures. In the series *a* the development proceeded for five hours, and in *b* for twenty-four hours at 20° C. $\frac{400}{1}$. (After Klöcker and Schiönning.)

g. 130 *d*), while the intrusive mycelium thread forms idia (Fig. 130 *b*). When a weak cell lies between two orous ones, both of these latter can grow into the feeble and form chains of yeast conidia (Fig. 131 a_1-a_4), or one intrudes a mycelium thread, while the other simply forms conidia (Fig. 130 *e*). Fig. 131 shows two developmental series of such endogenous conidia, and Fig. 132 the germination of the same. As with *Oidium*, the phenomenon appears when young, vigorous mycelium is seeded in a little water; when placed in wort or on moist gypsum blocks, the mycelium can also display inter-growth, but it does not take place nearly so frequently.

This fungus forms a very strong layer on nutrient liquids. v. Skerst observed the following limits of temperature for its growth in wort: Maximum 31° to 32° C., optimum 16° C., and minimum 0·5° to 2° C. In a related form Hoffmann had found that the gelatinised cell walls were stained blue by iodine. The author observed that this also occurs sometimes in the typical *Dematium pullulans*.

The fungus is extremely common in nature, especially on fruit. It is found in moist places in breweries.

It decolorises wort and makes it ropy. According to Lindner it clouds white beer wort; according to Wortmann it also transforms grape must into a thready gelatinous substance, which is derived from a product of the outer parts of the cell membrane. This appearance is especially marked when cane sugar is present in the liquid. In wine, however, the fungus is quickly suppressed. though not killed, by the evolution of carbonic acid. In the presence of about 8 vol. per cent. of alcohol it does not grow, but is also not killed. The so-called cork or stopper flavour of wine arises from the cork of the bottle being overgrown with various fungi, *Dematium* and others.

According to Wortmann, *Dematium pullulans* is the cause of a disease of wine grapes. The attacked grapes look like those affected with "schwarzen Brenner," but the black spots are soft, not brittle, and depressed; further they have a white, mealy spot in the middle. At this place the mycelium of the fungus breaks through the epidermis of the grapes, and the yeast buds appear. The spots often reach half round the grapes.

Dematium pullulans is one of the mould fungi which has been repeatedly regarded as the original form of the saccharomycetes. Communications relative to this have, however, suffered the same fate as those in which the same is maintained for *Penicillium*, *Aspergillus*, *Mucor*, etc. ; as soon as exact experiments were made, these ideas were found to be quite incorrect.

Finally, it may also be mentioned that various Ascomycetes are found, *e.g.*, *Sphærulina intermixta*, Berk. and Br., and *Dothidea ribesia*, Pers., which can produce *Dematium*-like growths. If Brefeld says that the species in question produce *Dematium pullulans*, he is in error, because these growths do not entirely correspond with the species named. Moreover, Brefeld has also been unable to produce Ascomycetes from a typical *Dematium pullulans*. Under certain conditions it is also found that some of the forms of *Cladosporium herbarum* produce *Dematium*-like growths.

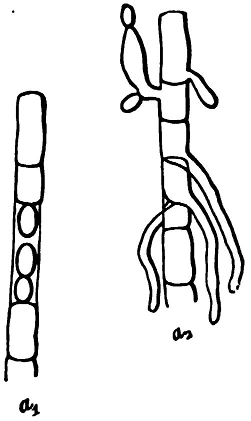


FIG. 132.—*Dematium pullulans*, De Bary. Germination of endogenous conidia in wort in a cover glass preparation. The conidia (a_1) swelled up after twenty-four hours and put forth germ threads (a_2) ; the same has also taken place with the uppermost two cells in the mycelium thread. $\frac{500}{1}$. (After Klöcker and Schiönning.)

Cladosporium herbarum, Link, is, as already mentioned, a collective name. In addition to the conidial form of *Sphærella Tulasnei*, referred to on p. 283, a number of other fungi are found, which have been classed under the name *Cladosporium herbarum ;* none of these, however, is known to possess ascus fructification. Some are similar to *Dematium pullulans*, and some investigators (*e.g.*, Laurent) have, for this reason, classed *Cladosp. herbarum* and *Demat. pullulans* in the same development series. Lopriore has studied a parasitical *Cladosporium herbarum* form occurring on wheat, which forms sclerotia. He states that it produces *Hormodendron cladosporioides* as well as *Demat. pullulans*. It is therewith stated that several species occur that produce conidial forms, which can be identified with the species referred to above.

II.—FISSION FUNGI (SCHIZOMYCETES).

General.

1.—STRUCTURE AND FORM OF BACTERIAL CELLS.

The Cell Contents.—Like yeast cells, the bacterial cell consists of a mass of protoplasm surrounded by a membrane. It has been contended as to whether it contains a cell nucleus or not. Later investigations, however, show that in this respect also bacterial cells resemble other cells.

As with yeast cells, vacuoles and granules occur in the protoplasm. In some bacteria a substance is found which is probably granulose or an allied carbohydrate, as it is stained blue by iodine. Sulphur granules or oxide of iron are found in other forms; occasionally fat globules are observed, especially in the cells of old cultures. Colouring matter is found in many species, often in such quantity that the colonies are highly coloured, *e.g.*, red, yellow, blue, violet, brown, etc. The colouring matter is found partly in the interior of the cells, and partly secreted as granules lying between the cells.

The Cell Wall and its Gelatinous Formation.—If the cell is placed in a solution of common salt, the plasma separates from the membrane so that the latter becomes plainly visible. This phenomenon is called plasmolysis. The membrane does not consist of cellulose but of albuminoids, probably modifications of those forming the protoplasm. The membrane often possesses the property of forming gelatine and swells up. The growths are then enveloped in mucilage and form gelatinous films or masses, called Zoogloeæ (Fig. 133). In some species the mucilage is stained blue by iodine, in others it gives the cellulose reaction (blue stain by iodine in zinc chloride or iodine and sulphuric acid).

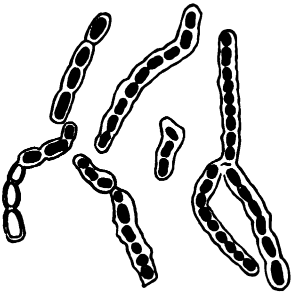


FIG. 133.—*Bacterium Pasteurianum*, Hansen. Gelatinous formation in an old growth on beer. The three cells to the left have fallen out. The cells are prepared and stained by Löffler's method. $\frac{1000}{1}$. (After Hansen.)

Flagella. — Many bacteria are observed under the microscope to be in motion; this motion is often due to physical causes and is then the so-called Brownian molecular movement. Another kind of movement is effected by special organs of motion, flagella or cilia. The rapidity which this motion attains, has, with some species, an average value of about $\frac{1}{9}$ mm. per second. The flagella or cilia were discovered in 1836 by Ehrenberg, and, as a rule, can only be observed after a special preparation (see p. 91). In some forms they are found at the poles of the cells, in others at the sides: occasionally several are found together. Alfr. Fischer distinguishes between bacteria (1) with one flagellum at the end (Fig. 135), (2) with a cluster of flagella at the end, and (3) with flagella over the whole surface (Fig. 134). Flagella furnish important characteristics for the determination of species, and for classification it is of importance to determine if the species concerned occurs with flagella or without.

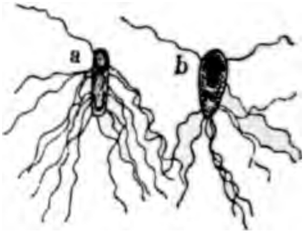


Fig. 134. *Clostridium butyricum*, Prazmowski. Butyric acid bacterium with flagella. *a*, Vegetative motile cell; *b*, sporulating motile cell. $\frac{1250}{1}$. (After Alfr. Fischer.)

Bacteria with flagella movement are called motile. This motion may be temporarily stopped by certain means, *e.g.*, by an increase in the acid content of the nutrient medium or by a deficiency of oxygen; the condition of the organism is then known as flagella-stiffness (Geisselstarre). By neutralisation or by aëration the stiffness can again be removed. When, therefore, bacteria in a culture do not move just at the instant, one cannot be always certain that the power of motion has altogether left them. Various sub-

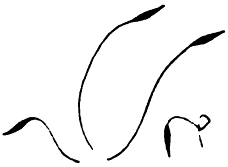


Fig. 135. *Termobacterium aceti*, Zeidler. Acetic acid bacterium with one flagellum. $\frac{1200}{1}$. (After Zeidler.)

stances can attract or repel motile bacteria; this property is known as positive or negative Chemotaxis.

Shape of the Cell.—Some of the cell shapes which occur most frequently are as follows: Spherical bacteria are known as cocci (Fig. 136, A), rod-shaped ones, *i.e.*, those which are at least double as long as they are broad, as bacilli or long rods (Fig. 136, B, 1, 2, 4, 5), and those of which the length and breadth are more nearly equal, as bacteria

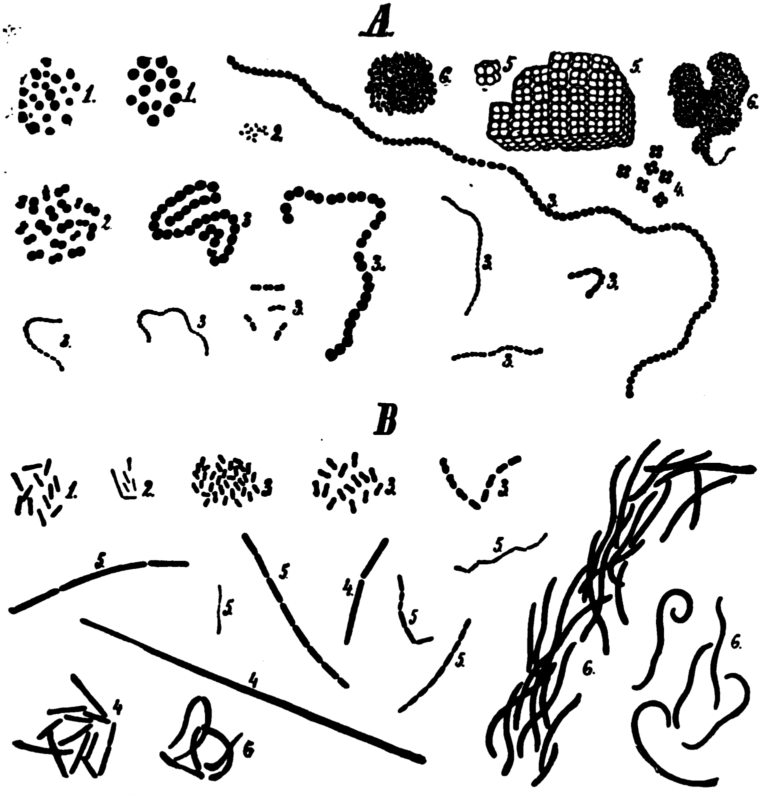


FIG. 136.—Form and size of various bacteria. A, 1. *Micrococcus* of various sizes. 2. Diplococci. 3. Streptococci. 4. *Micrococcus tetragonus.* 5. *Sarcina ventriculi*, package form. 6. Staphylococci. *B*, 1, 2, 4. Long rods. 3. Short rods. 5. Connected chains of long and short rods. 6. Long threads. $\frac{900}{1}$. (*A*, 5. $\frac{1200}{1}$.) (After P. Baumgarten.)

(in the true sense) or short rods (Fig. 136, B, 3, 5). If a *Bacillus* displays (as the result of spore formation) a more or less decided club shape it is called *Clostridium* (Fig. 137); if curved it is called *Vibrio*, and if it is screw shaped, *Spirillum*. The very long thread-like bacteria are known as *Cladothrix*, *Streptothrix*, *Crenothrix*, etc. It is common for the same species to occur in different forms.

Most bacteria are very small, and a powerful magnification is required in order to observe them. The smallest cells are not more than 1 μ long. Especially large forms are, *e.g.*, *Bacillus oxalaticus*, whose rods may be 30 μ long and 4 μ thick, and *Bacterium megatherium*, whose rods are 10 μ long and 2·5 μ broad.

2.—METHODS OF REPRODUCTION.

Cell Fission. — As above mentioned, the vegetative multiplication of bacteria proceeds by division or fission; many forms have only this one method of multiplication, whilst others can, in addition, form endospores. Rod bacteria become elongated, and divide by means of a septum; the latter never occurs lengthways; spherical bacteria do not alter their original appearance before division has taken place. In the latter, division can take place in several directions. The new-formed cells often remain in union with the mother cells, whereby long chains may be formed.

By seeding on various nutrient gelatines the species produce colonies, which have a more or less varied macroscopical appearance, as regards both shape and condition of the surface (slimy, dry). In this connection the method by which the colonies were started is also of influence, whether it was by stab or streak cultures or giant colonies from the seeding of a drop. It has already been said that the colour of the colonies may vary.

If a nutrient solution remains clear after seeding, the species present is one with pronounced chain or thread growth and without self-movement. Forms which require oxygen for their growth develop a film on the surface of the, as a rule, clear liquid. If the latter is uniformly turbid, the forms are isolated, living and sometimes motile; in this case also the oxygen-absorbing species form a film.

Spore Formation.—Endospores are seen as strongly refractive granules in the interior of the cells; usually each cell contains one spore, seldom two. They are formed by the contraction of the contents which become gradually thicker and more refractive and, finally, bounded by an independent wall. The latter is usually smooth; only one species is known in which a membrane of special structure with longitudinal ridges is to be observed (Arth. Meyer). With certain species the cell assumes a special shape during spore formation, becoming, for example, spindle-shaped (Fig. 137, B, *f*, *h*). The spore has a much greater power of resistance to external influences than the vegetative cell; aniline dyes are taken up by it much more slowly than by the remaining plasma; on the other hand these colours are obstinately retained when a decolorising reagent is afterwards employed. Extreme degrees of cold and heat, besides complete drying, are endured by the spores without death ensuing. On the contrary, light appears, in the generality of cases, to be the deadliest enemy of bacterial spores. The spores of several species resist a boiling temperature for some hours; their germinating power may even be increased by this means.

Through the great resisting power of bacterial spores, which is more marked in the dry than in the moist state, sterilisation is found to be difficult in many cases, *e.g.*, in order to sterilise water it must be boiled under pressure, or, if this is not convenient, raised to boiling temperature several times at intervals (see p. 78). Wort is often not sterile after boiling; that this is the case can be seen if a sample is added to yeast water, when frequently a growth of bacteria makes its appearance. That no growth appears in flasks of wort after simple boiling may be due to the fact that the culture medium is not favourable for the germination of the spores present. Wort is indeed a liquid in which many species of bacteria, even in vigorous condition, are not able to grow.

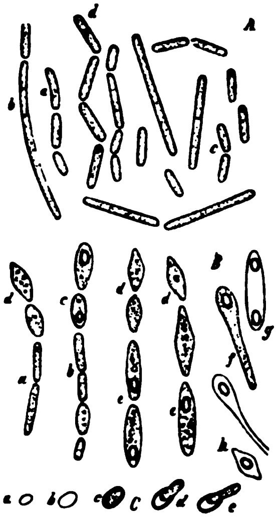


FIG. 137.—*Clostridum butyricum*, Prazmowski. A, Vegetative cells. B, Spore-bearing cells, with the exception of *a* and *b*. In *f* and *h*, the cells are swollen to spindle shapes, the formation of the spore being here complete. Two spores have grown in *g*. C, Germinating spores. (After Prazmowski.)

As soon as the spore is ripe it is able, under favourable conditions, to germinate. Before germination proceeds the spore swells out and thereby loses its brilliancy, the skin then bursting and a small knob protruding. This protrusion can take place either at the pole of the spore (Fig. 137, C), the new cell continuing its growth lengthwise from the spore, or the spore skin bursts in the middle and the young cell grows direction perpendicular to the longer axis of the spore. se are the two commonest types of germination of bacl spores; and other ways are quite exceptional.

The causes of the production of spore formation are to sought in unfavourable nutrimental conditions, accumuon of injurious products of growth, etc. Like the charomycetes, certain bacterial forms require free access air in order to form spores.

3.—VARIATION.

Hansen's experiments with the acetic acid bacteria: *acterium aceti*, *Bact. Pasteurianum* and *Bact. Kützingianum*, ave shown that temperature is a factor for influencing hape. His conjecture that the results found by him have more general application was confirmed by the author's xperiments with four other species. Henneberg came to the same conclusion from experiments with *Bact. oxydans* and *Bact. acetosum.* The investigations of Hansen demonstrated that the species named appear with three different cell forms, *viz.*, sometimes as short rods in chains (Figs. 139 *a*, 144, 145 and 146), sometimes as threads (Figs. 138, 139 and 140), and at other times as pear-shaped or globular swellings (Fig. 140). When young, vigorous cells are seeded in a favourable culture medium rich in extract, *e.g.*, "double" beer (Danish beer with little alcohol and high extract) or wort, at a temperature between 5° and 34° C., the chain form with the short rods appears; if, now, such a growth be inoculated in the culture medium of a new flask and kept at 40° to 40½° C., the cells are quickly changed into threads (Figs. 138 and 139). The latter can attain a length of 500 μ, while the cell seeded only measured 2 μ. If the thread form be now brought again to 34° C., the length continues to increase; here and there globular, spindle, or pear-shaped swellings may occur, whereupon the threads begin to divide again and to assume the chain form (Fig. 140). By Nageli and others such swollen forms were regarded as not belonging to normal development, but as an indication that the cells are about to die. It is shown by Hansen's above-mentioned investigations that, under certain culture conditions, these peculiar forms appear normally and indicate a vigorous growth.

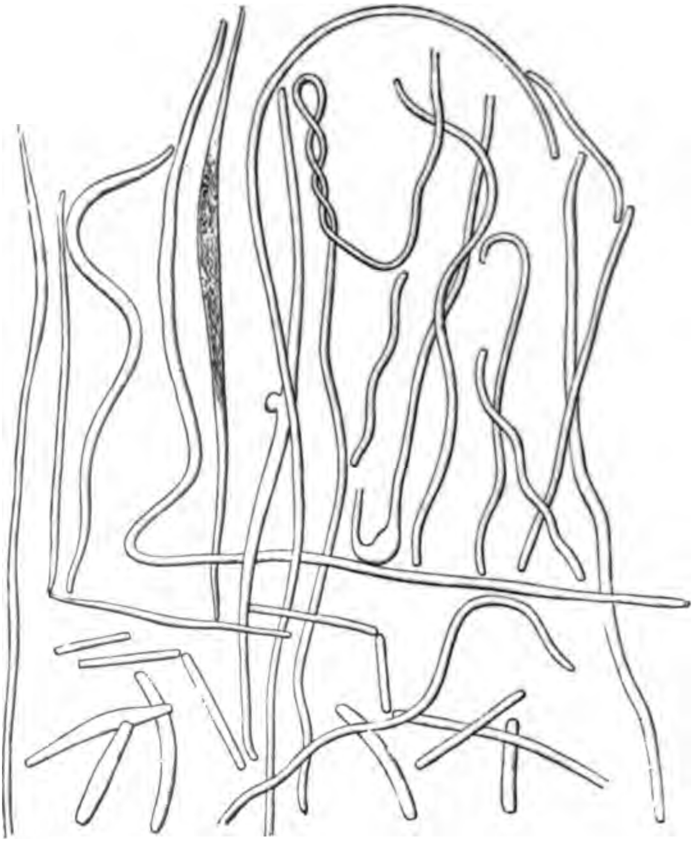


FIG. 138.—*Bacterium Pasteurianum*, Hansen. The thread form after twenty-four hours in "double" beer at 40° to 40½° C. $\frac{1000}{1}$. (After Hansen.)

Beyond the above-mentioned investigations on acetic d bacteria, only few and incomplete contributions to this bject are as yet to be found. Macfadyen and Blaxall and that some thermophilic bacteria investigated by them so formed long threads at high temperatures (approaching)° C.).

It is generally known that among organisms different onditions can produce identical or nearly corresponding forms of development. It is therefore not remarkable that various authors have stated that the change of shape referred to can also be obtained as the result of the influence of factors other than temperature; H. Buchner has found, in regard to *Bacillus subtilis*, that the chemical composition of the culture medium was responsible for similar changes of shape.

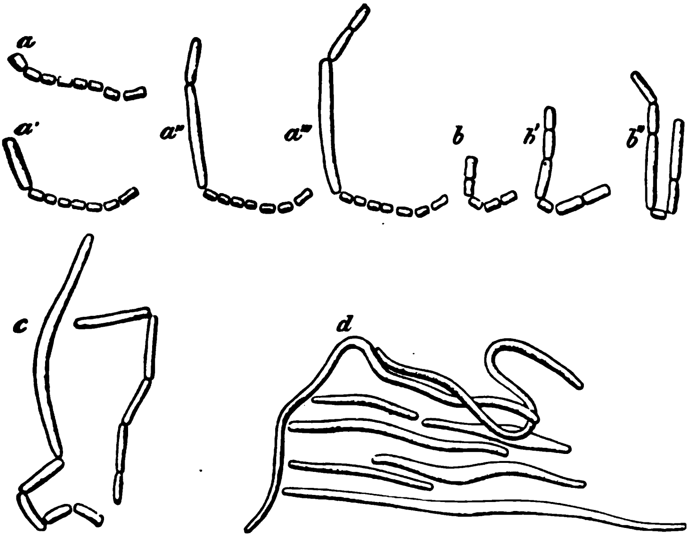


FIG. 139.—*Bacterium Pasteurianum*, Hansen. Development of the thread form by cultivation on "double" beer agar-agar in a Böttcher's chamber at about 40½° C. *a*, A chain consisting of 8 members; *a'*, the same after 6; *a''*, after 10; *a'''*, after 20 hours. *b*, A five-membered chain; *b'*, after 5; *b''*, after 9 hours. *c*, Development after 10; *d*, after 21 hours. The times are reckoned from the beginning of the experiment. $\frac{1000}{1}$. (After Hansen.)

The abnormal and in general inflated shapes assumed by many bacteria when they have to exist for a long time in unfavourable environments are usually known as involution forms. These may take up the most varied shapes.

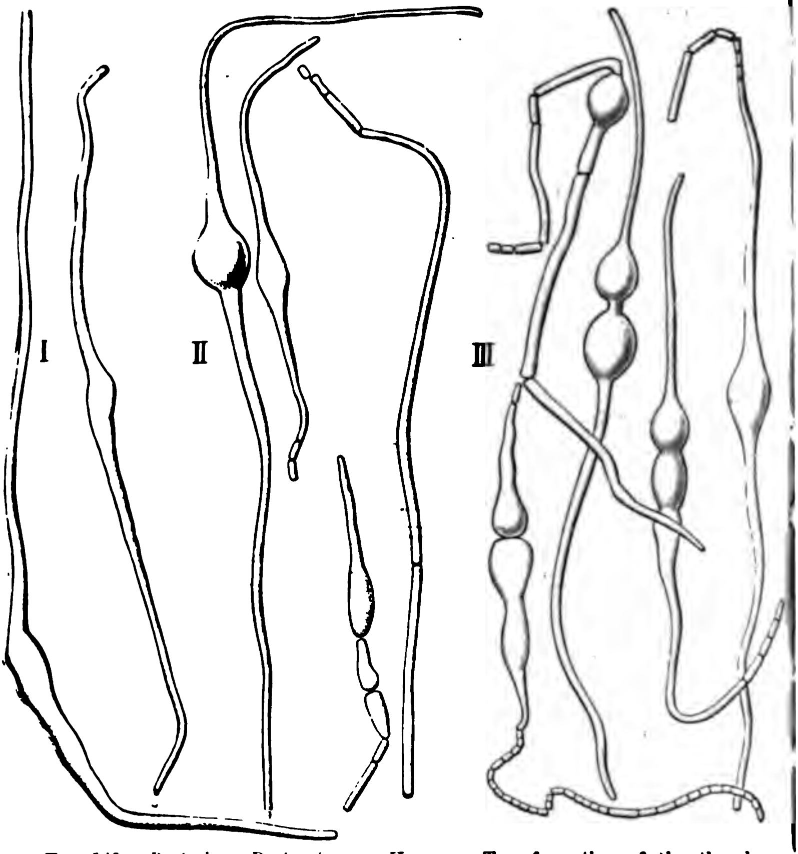


FIG. 140.—*Bacterium Pasteurianum*, Hansen. Transformation of the thread form into swellings and chains by cultivation in "double" beer at 34° C. I. after four, II. after five, III. after seven hours from the beginning of the experiment. $\frac{1000}{1}$. (After Hansen.)

Some, *e.g.*, the acetic acid bacteria (Fig. 141), the diphtheria bacillus, tubercle bacillus, etc., sometimes assume a mycelium-like, branched appearance, and it has been attempted, without grounds, however, to make use of this fact in order to identify the parent forms of bacteria among the hyphomycetes. They are, however, as stated, only abnormal formations. A bacterium has never been grown and developed into a hyphomyces, and *vice versâ.*

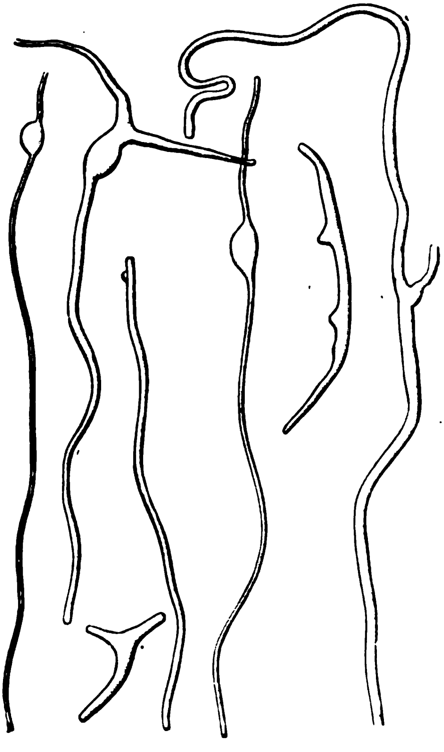


FIG. 141.—*Bacterium aceti* (Kützing), Hansen. Unusual cell forms after several days' cultivation in wort and "double" beer at 39° to 41° C. $\frac{1000}{1}$. (After Hansen.)

All communications which have hitherto appeared on the development of bacteria from higher fungi are without proof.

With many species an enfeeblement takes place during continued culture, so that, for instance, the fermentative activity becomes lost (as distinguished from the saccharomycetes, in which this has never been observed). Bacteria which cause diseases in man and animals, can lose their virulence (pathogenic properties) by means of a special treatment. A change of another kind has been likewise observed in the splenitis bacillus, which, during its temporary change of form, can lose the power of spore formation; this change of form displays itself in the production of rudimentary spores.

With regard to variation among vinegar bacteria, Hansen has further shown that the mucilage of his two species, *Bact. Pasteurianum* and *Bact. Kützingianum*, which is stained blue by iodine, loses this property under certain conditions. Whether developed on the surface of a liquid or solid medium, a time arrives when they no longer give the blue reaction. In some beer cultures he found that this point was reached in three to four months at the ordinary temperature of the room; in others, on the contrary, it was not even attained after seven to nine months. These abnormal cultures usually return quickly to the normal state.

4.—DISEASE BACTERIA IN THE ALCOHOLIC FERMENTATION INDUSTRIES.

The harmful influence of bacteria in the fermentation industries is by no means small; there are, however, only relatively few species which do an appreciable amount of damage. The majority, in fact, do not thrive, or do so but indifferently, in acid liquids; beer and wine contain free acid, and are thus protected to a great extent. By the introduction of the pure culture system this injurious action of bacteria has been very much lessened in bottom and top fermentation breweries, as well as in the manufacture of wine.

The decomposition products of bacteria are of many kinds. They can develop acetic acid, lactic acid, butyric acid, various alcohols, etc. A large number liquefy nutrient gelatine through the possession of peptonising power.

Pasteur had discovered in the year 1861 that butyric acid bacteria are anaërobic, that is, they can only grow in the absence of oxygen. The majority of species, however, thrive best with free access of air. The acetic acid bacteria are typical examples of aërobic forms.

Pasteur (1876) was also the first to point out the injurious part played by bacteria in the fermentation industry. The disease phenomena brought about by bacteria in fermented liquids are: mucilage formation, decolorisation, turbidity, acid formation, disagreeable smell and taste.

Pasteur gives some information on mucilage formation in beer and wine; he mentions a *Micrococcus* which makes the liquids mentioned ropy. Kramer isolated from thick wines a *Bacillus* with which he could induce the same disease in sound white wine. From ropy Belgian beers, van Laer isolated bacilli which were the cause of the disease. L. Vandam also found in English beer a *Bacillus* that makes it thick. At the same time this species only attacks the beer when present in large number at the beginning of the primary fermentation. Brown and Morris describe a *Coccus*, likewise causing ropiness in English beer. The disease attacked the beer when it was six weeks to two months old. The source of infection appeared to be a slaughterhouse for pigs in the neighbourhood of the brewery. A *Pediococcus* was found by Lindner in ropy "Weissbier".

The disease known as the turning of wine consists in red wine assuming a brown colour, while white wine becomes turbid and discoloured and frequently develops a dark colouration. The tartar occurring in wine is changed by this disease into potassium carbonate, which causes the [illegible] Kramer named two [illegible] species [illegible] wines.

The bacteria of lactic acid, butyric acid and acetic acid [illegible] acid producers in fermented liquids. [illegible] the first are especially active in the mashing stage, [illegible] in the lager a species, Saccharobacillus [illegible] causes the turning of beer. The beer thereby [illegible] becomes disagreeable in smell and taste [illegible]. The [illegible] consists partly of nitrogenous [illegible] which separate as a result of lactic acid production and partly of the bacteria themselves. Butyric acid [illegible] causes the "Zickenwerden" (see [illegible] wine). Butyric acid bacteria are responsible for a [illegible] smell and taste in beer. They are especially [illegible] in distilleries.

Acetic acid bacteria are found particularly in wine [illegible], where they occasion the tartness of the wine. [illegible] the wine has been strongly attacked by them, [illegible] vinegar sour, it is valueless; there is [illegible] of remedying the evil. In brewing they are [illegible] in top fermentation breweries, where the conditions for their development are more favourable than in bottom fermentation breweries. Hansen experimented in practice with Bact. aceti and Bact. *Pasteurianum* in the presence of Carlsberg bottom yeasts No. 2 and No. 1. He came to the conclusion that the bacteria in question were indeed present in the finished lager beer if the infection took place at the beginning as well as at the end of the primary fermentation, but that this infection appeared neither in the fermentation cellar nor in the lager cellar. The bacteria can propagate themselves only after the lagered beer is drawn off; the beer, however, did not become vine-ar sour if care was taken that the transport vessels and ttles were well closed and well filled. The same holds good also when the bacteria are introduced into bottom fermented beer after it has left the lager cellar. The infection generally occasioned no bad effects when the precautions named were observed. High temperature and free air access are necessary for the development of the bacteria.

Gayon and Dubourg found in wine containing mannite, a short non-motile rod which forms masses of zoogloea. It reduces invert sugar to mannite. Wine, however, is most exposed to this disease in warm climates. (As formerly mentioned, *Penicillium glaucum* can cause the same phenomenon.)

According to the investigations of V. H. and L. J. Veley, the disease of rum, called "faultiness," is brought about by a bacterium which these authors name *Coleothrix methystes*. This species is especially distinguished by its great power of resisting alcohol, the bacteria retaining life in rum with an alcohol content of 75 per cent. by weight.

Pediococcus or *Sarcina* species frequently occur in breweries. However, only certain of these cause harm, and these only under special conditions. It has been shown by Hansen, Jörgensen and Ant. Petersen that when certain of the species occur even in considerable amount in beer, they are without appreciable influence on its quality. These forms have been especially studied by P. Lindner (1888). According to him and other authors, some species under certain conditions occasion "sarcina turbidity". In this connection Reichard states that the disease appears when a strong after-fermentation takes place in beer inoculated with *Sarcina*, while equally strongly infected beer did not succumb to the disease when the primary fermentation was a strong one, so that in place of the vigorous after-fermentation, only a slight maturing took place. An addition of hops to the lager casks will, according to the same author, prevent an outbreak of the *Sarcina* disease.

Schönfeld has also investigated the infection of yeast by *Pediococcus* and *Sarcina*. His results are the following: Culture yeasts are susceptible to *Sarcina* in varying degree. Wild yeasts are more prejudicial to the development of *Sarcina* than culture yeasts; with favourable conditions a *Sarcina* acclimatised to any yeast race can spoil the beer prepared by means of this yeast; *Sarcina* is more anaërobic than aerobic: the virulence of *Sarcina* is dependent upon certain medium temperatures, an absence of air, and on motion; the multiplication of *Sarcina* is restricted by strongly hopping the beer, by high alcohol content and by aeration.

Kupfer seeks for the causes of the poor "head" and slight stability of beer in *Sarcina* infection.

S. v. Huth has recommended the employment of a tartaric acid remedy for *Sarcina*, in which 6 grams of tartaric acid are added in aqueous solution to every kilo of yeast. After stirring, the whole is put on one side to rest for six to twelve hours, after which the mixture is introduced into the fermenting vessel. This method must always be applied with care, and is only to be recommended when the stock yeast contains practically no wild yeast. For if this is not the case the latter develops at the expense of the culture yeast, and the result proves correspondingly unfavourable.

5.—APPLICATION OF BACTERIA IN THE ALCOHOLIC FERMENTATION INDUSTRIES.

Only the lactic acid-forming bacteria are directly employed in the alcoholic fermentation industries, these being applied in distilleries in subduing butyric acid bacteria and in the "Weissbier" breweries. In breweries the yeast settles to the bottom of the dilute wort; such does not happen in distilleries, where the thick mash does not allow of this.

The pitching yeast is therefore produced by itself in special vessels. The requisite amount of nutrient liquid for this is acidified in order to prevent the development of harmful germs, such as butyric acid bacteria, which are unable to thrive in strongly acid liquids. A strong and suitable formation of acid is therefore effected by the addition of a young and vigorous pure culture of lactic acid bacteria. The latter is obtained by starting from a trial mash of which the souring is satisfactory. The optimum temperature for lactic acid bacteria lies at about 50° C., that of butyric acid bacteria, on the other hand, at about 40° C. The sweet yeast mash is therefore kept at about 50° C., in which case only a strong growth of lactic acid bacteria develops. When the degree of acidity is sufficiently high, the lactic acid bacteria are killed by warming the mash up to 70° C., following this it is quickly cooled to 17° to 20° C., and a pure culture of a selected yeast race added. A part of the yeast produced is then taken out to be used in pitching a fresh portion of sweet mash.

As formerly mentioned, F. Lafar was the first to introduce the systematic selection of a lactic acid bacterium for this purpose. Wehmer, however, has lately attempted to suppress butyric acid bacteria with commercial lactic acid, which, according to his investigations, has given good results.

Of the remaining bacteria referred to, those of acetic acid are employed, as is well known, in the manufacture of vinegar.

SYSTEMATIC.

Ferd. Cohn showed that bacteria are plants, and set up a system based on cell form. We have seen, however, that the same species can assume various forms of growth.

For the investigation of different species, the morphological and developmental characteristics are, in the first place, applied as far as possible, *e.g.*, the shape of the cell, the presence of flagella and their arrangement, the mode of germination of the endospores, etc. Further, the forms which the growth assumes on various nutrient media, *e.g.*, on culture gelatine, in which case it is also observed to what extent the species liquefies gelatine, if at all. The products resulting from growth are also of importance. The cultures are prepared either as streak or stab cultures, the former on the surface of the gelatine, the latter by stabbing in a thick layer of gelatine (see p. 98). Drop seeding on the surface of the gelatine is also employed. The behaviour of bacteria species with various staining methods takes a large place, especially in medical bacteriology.

The bacteria considered here can be divided into two chief groups, *viz.*, spherical bacteria (*Coccaceæ*) and rod bacteria (*Bacteriaceæ*), by which means, on practical grounds, we retain the old classification, in which shape was the criterion, and also the older names which are generally known. The author, however, is, on this point, neither for nor against this arrangement. The classification of bacteria is at present very uncertain, and is still subject to change, since no fixed, systematic, distinctive lines can be laid down. That the shape, moreover, is in several respects of little value as a systematic distinguishing feature follows, among other things, from what has been said in the foregoing on the polymorphism of bacteria.

I.—FAMILY: SPHERICAL BACTERIA (COCCACEÆ).

In the isolated state the cells are spherical. Fission takes place along one, two or three planes.

1. *Genus : Micrococcus, Cohn.*

The cells are joined together irregularly. Included in is genus are several forms which, like *Sarcina*, divide ong three planes, but the cells quickly separate from one nother and do not remain united as in *Sarcina*. Such pecies ought rightly to be classified in the genus named.

Micrococcus viscosus is, according to Pasteur, the cause f the ropiness of beer.

Micrococcus saprogenes vini I. and II., Kramer, has been obtained by Kramer in pure cultures from turned wines from Styria and Croatia.

Diplococcus I. and II., Aderhold, makes wine ropy.

2. *Genus : Pediococcus, Francke.*

The cells are arranged in flat colonies; they divide along two planes.

Pediococcus cerevisiæ, Francke, was first prepared as a pure culture by P. Lindner. The cells are 0·9 to 1·5 μ in diameter. They occur either as coccus or diplococcus or in tetrads, and are to be found in large quantities in beers with the so-called sarcina turbidity.

Pediococcus viscosus, Lindner, has been isolated by P. Lindner from thick "Weissbier."

Pediococcus sarcinæformis, Reichard, causes disease in beer, according to Reichard.

Pediococcus acidi lactici, Lindner. The cells are 0·6 to 1·0 μ in diameter. This species excites lactic acid fermentation in malt mashes. The optimum temperature for its growth is 40° C., whilst it dies at 62° C. It does not thrive in hopped wort nor in beer.

3. *Genus : Sarcina, Goods.*

The cells are arranged in packet-formed octads (Fig. 136, A, *b*); they are divided by three fission planes. To thi genus belong species which form white, yellow, red or brown colonies.

Sarcina maxima, Lindner, is found in malt mashes. The cells are 3 to 4 μ in diameter.

Sarcina aurantiaca, Lindner, has been isolated from Berlin "Weissbier". This species, as also *S. flava* and *S. alba*, is said to give rise to diseases in American beer.

The species belonging to the two foregoing genera are very common in nature, and occur, among others, also in water. Jorgensen and Lindner, for example, found these forms in their water analyses. Their occurrence in breweries has been mentioned above.

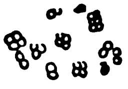


FIG. 142.—*Sarcina.* (After Hansen.)

II.—FAMILY: ROD BACTERIA (BACTERIACEÆ).

The cells are of various lengths, cylindrical and straight never spiral. Fission takes place only in one direction, *viz.*, transversely, and after previous elongation of the rod.

Genus 1 *and* 2: *Bacterium and Bacillus.*

The older systematists, Cohn for example, called all short rods *Bacteria*, all long rods *Bacilli*. At present it is impossible to separate the two genera completely from one another by this means. For the species appear sometimes as short, sometimes as long rods. The author, however, retains both these generic names on practical grounds, the species being hence represented with their old names; so that, for example, the generic names *Granulobacter*, *Clostridium*, etc., are retained in what follows. For distinguishing purposes the spore formation and the flagella have been suggested; but these characteristics also have proved to be insufficient.

Acetic Acid Bacteria.

Kützing (1837) first described an acetic acid bacterium, whilst Hansen was the first to show that various species of acetic bacteria exist, and to give at the same time the outlines of their life history (1879). The investigations of A. J. Brown, Henneberg, Zeidler and Zopf show that a few of these species have a swarming state under certain conditions. One of these acetic acid bacteria with flagella is shown in Fig. 135. In this case the flagella are of particular importance in characterising the species. With some species the mucilage which is formed by the cells gives a blue reaction with iodine (Hansen), that of others yields the cellulose reaction (a blue stain with iodine and sulphuric acid; A. J. Brown).

Hansen has given information on the vitality of these bacteria in different nutrient media and also in the dry state. He found that *Bacterium aceti* lived in "double" beer more than six years, in some cases, however, not five years; further, that after nine years in lager beer and after about two years in a saccharose solution it was still alive; *Bacterium Pasteurianum* was alive after six years (in one case it was dead after two and a quarter years) in "double" beer, after more than ten years (in one case death occurred after one to two years) in lager beer, and after one year and a quarter in a saccharose solution; but in the latter it was dead after one year and a half; lastly, *Bacterium Kützingianum* was alive after about six years in "double" beer (in some cases it was dead after five years), and after about seven years in lager beer (in some cases after five years only). In the dry state on small pieces of platinum wire in Freudenreich flasks the life limit of the cells of the three species named was about five months. When the above-mentioned dry preparations were introduced into glass tubes, and these closed by fusing, and preserved at the ordinary room temperature and at 2° C., it appeared that the lifetime at the former named temperature was about five months, and more than a year at the latter. When the cells were introduced into the tubes in a moist state they very soon died.

The formation of acetic acid induced by these organisms is brought about by the alcohol being converted into acetic acid in the presence of a plentiful air supply. As Pasteur showed (1864), and A. J. Brown confirmed later, the combustion can proceed still further, so that the acetic acid already formed is oxidised to carbonic acid and water.

F. Lafar studied the influence of temperature on *Bact. aceti* and *Bact. Pasteurianum* with regard to their power of forming acetic acid. He found that the former species at as low a temperature as 4° to 5° C., the limit of its growth, can set up a strong acetic fermentation, but that *Bact. Pasteurianum*, on the other hand, formed no acetic acid at 4° to 4½° C. With the last-named species he found, further, that the maximum acid formation is reached at 33° to 34° C. after seven days, when 3·3 per cent. by weight is produced. W. Seifert came likewise to the conclusion that morphologically different species of acetic acid bacteria also exhibit substantial differences in regard to their chemical behaviour. In his experiments he employed *Bact. Pasteurianum* and *Bact. Kützingianum*. He draws the conclusion, "that the fermentative power of acetic acid bacteria in the presence of the monatomic primary alcohols decreases as the proportion of carbon in the latter increases; that, further, *Bact. Pasteurianum* has the feeblest fermentative power in the presence of the polyatomic alcohols and dextrose". Henneberg carried out investigations similar to Seifert's, but with a whole series of species of which some are new: he arrived at the same general result.

In the following table from Henneberg will be found numerous substances which can be converted into acids by the various species. The sign + indicates that acidification, — that no acidification takes place.

	Bact. industrium.	Bact. oxydans.	Termo-bact. aceti.	Bact. aceti.	Bact. acetosum.	Bact. Kützingianum.	Bact. Pasteurianum.	Bact. acetigenum.	Bact. ascendens.	Bact. xylinum.
Arabinose . . .	+	+	–	–	–	–	–	–	–	–
Levulose . . .	+	+	–	–	–	–	–	–	–	–
Dextrose . . .	+	+	+	+	+	+	+	+	–	+
Galactose . . .	+	+	–	–	+	–	–	–	–	–
Saccharose . .	+	+	–	–	–	–	–	–	–	slight
Maltose . . .	+	+	–	–	–	–	–	–	–	–
Lactose . . .	+	slight	–	–	–	–	–	–	–	–
Raffinose . . .	+	+	–	–	–	–	–	–	–	–
Dextrin . . .	+	+	–	–	–	–	–	–	–	–
Starch . . .	+	–	–	–	–	–	–	–	–	–
Ethyl alcohol .	+	+	+	+	+	+	+	+	+	+
Propyl alcohol .	+	+	+	+	+	+	+	+	+	+
Glycol . . .	+	+	+	+	+	+	+	+	+	+
Glycerine . .	+	+	–	–	–	–	–	–	–	
Erythrite . .		+		–	–	–	–	–		
Mannite . . .	+	+	–	–	–	–	–	–	–	–

The variation in shape with the same species, according as the temperature lies near the optimum or the maximum, has been referred to previously (p. 319; *cf.* Figs. 138, 139 and 140), and also the variation of the iodine reaction of the mucilage.

We have also seen that acetic acid bacteria cause the sharpness of wine and that they only give rise to loss in breweries when a high temperature and free air access are obtainable.

Pasteur (1868) was of the opinion that acetic fermentation was caused by only one species. He worked out a new method for the manufacture of vinegar, to displace the old Orleans method in which the fermentation proceeded slowly in barrels. As these barrels were employed for several years without emptying and cleansing, large quantities of *Vibrio aceti* were frequently developed, which caused considerable trouble. The Pasteur method consisted in using shallow vessels instead of barrels; he thus endeavoured to provide the most favourable conditions for the development of the film of acetic bacteria and by this means to bring about as quickly as possible the formation of vinegar so that the *Vibrio aceti* could not develop. For various reasons the method did not, however, obtain a foothold in practice, partly because the results obtained were so uncertain since there was no question of the application of a selected species or race. In the first edition of his *Untersuchungen aus der Praxis der Gärungsindustrie*, Hansen had, in this connection, already directed attention to the problem of also applying the pure culture system to the manufacture of vinegar; but only of late has the way been paved for reform in this direction by the researches of the experimental station in Berlin; the goal has not yet been reached, however. The question here concerns the most important method of vinegar manufacture, *i.e.*, the so-called quick vinegar manufacture of Schützenbach. The acetic bacteria occurring in this connection will be described later.

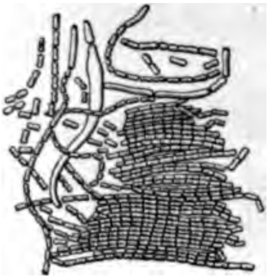


FIG. 143.—*Bacterium aceti* (Kütz.), Hansen. Young film formation on “double beer at 34° C. $\frac{1000}{1}$. (After Hansen.)

Acetic bacteria naturally fall into groups according as they possess flagella or not, and according as their mucilage gives a blue reaction with iodine or not. In the following it is expressly stated if the species in question has flagella or gives the blue iodine reaction. The first three following, the descriptions of which are from Hansen, belong to species without flagella.

Bacterium Aceti (Kütz.), Hansen (Figs. 141, 143).—This species forms a smooth gelatinous film on “ double ” beer at

C. after twenty-four hours. The film cells are usually [illegible]r-glass shaped rod bacteria arranged in chains (Fig. [illegible]); long rods and threads with or without swellings are [illegible]y exceptionally found. At 40° to 40½° C. long thin [illegible]eads develop. The mucilage is not stained by a solution [illegible] iodine or by a solution of iodine in potassium iodide. [illegible]fter four days at 25° C. it develops colonies on wort [illegible]latine which are grey, waxy, round, usually arched and [illegible]ith unbroken edges, seldom star-shaped, and which consist [illegible]hiefly of free, small rod bacteria; the chain form is repressed [illegible]ere. The maximum temperature of growth in "double" [illegible]eer is about 42° C., the minimum 4° to 5° C.

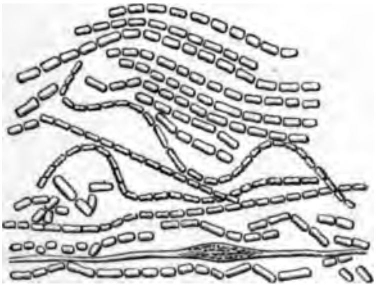


Fig. 144.—*Bacterium Pasteurianum*, Hansen. Young film formation on "double" beer at 34° C. $\frac{1000}{1}$. (After Hansen.)

The species is found both in top fermentation and in bottom fermentation beers; Hansen observed it frequently in the dust of the air and Holm now and then in his analyses of water.

Bacterium Pasteurianum, Hansen (Figs. 133, 138, 139, 140 and 144).—This bacterium forms on "double" beer at 34° C. after twenty-four hours a dry film which very soon assumes a wrinkled and folded appearance, and rises but little from the surface of the liquid along the sides of the flask. Like those of the foregoing species, the cells are arranged in long chains, but are altogether larger and especially thicker

(Fig. 144). The thread form (Figs. 138, 139 and 140) is also somewhat thicker at 40° to 40½° C. than with *Bact. aceti*. The mucilage is stained blue with a solution of iodine or of iodine in potassium iodide. Plate culture colonies on wort gelatine after four days at 25° C. are usually smaller than those of *Bact. aceti*, otherwise they are the same as with this species. They consist chiefly of typical chains. After about three weeks the surface of the colonies is folded. On wort gelatine and on lager beer gelatine, with and without 10 per cent. of saccharose, the growth is, at 25° C., solid, dry, waxy and yellowish. The maximum temperature for growth on "double" beer is 42° C., the minimum, 5° to 6° C.

Hansen found this species in the same places as the previous one, but more frequently in top fermentation than in bottom fermentation breweries. W. Seifert found it in vinegar-tainted wine.

Bacterium Kützingianum, Hansen (Fig. 145).—The film formed on "double" beer after twenty-four hours at 34° C. differs from that of *Bact. Pasteurianum* by growing high above the liquid along the sides of the flask. It consists of small rod bacteria which are, as a rule, free, or at most joined in pairs; they form long chains rarely. The thread form at 40° to 40½° C. contains relatively more short threads than *Bact. Pasteurianum*. The microscopical appearance differs distinctly from that of the latter; on the other hand, both species are alike in that the mucilage is stained blue by a solution of iodine or of iodine in potassium iodide. In plate cultures on wort gelatine this species develops colonies after four days at 25° C., which consist almost exclusively of small, free, rod bacteria; long chains are found only very seldom. The surface of the colonies after three weeks is smooth, not folded. The growths at 25° C., both on lager beer gelatine and wort gelatine, with and without 10 per cent. of saccharose, are shiny, slimy and bluish-grey. The maximum temperature of growth is about 42° C. in "double" beer, the minimum, 6° to 7° C.

Hansen found this bacterium in "double" beer; it i probably diffused to the same extent as *Bact. Pasteurianum.*

In addition to the above-cited differentiation between *Bact. Pasteurianum* and *Bact. Kützingianum* furnished by Hansen, Henneberg and Seifert have given an extensive series of physiological characteristics, among which, for example, are the following: *Bact. Pasteurianum* forms almost half as much acetic acid as *Bact. Kützingianum* in lager beer with added alcohol. Thus the former, in lager beer, with 8 per cent. of alcohol, produces 3·23 per cent. of acetic acid in twelve days, while the latter forms 6·56 per cent.; the formation of acid by *Bact. Pasteurianum* is likewise only half as great as that by *Bact. Kützingianum* when the cultivation takes place in a 2 per cent. glycol solution. When yeast water with 3 per cent. of dextrose is used as the nutrient solution, the growth of *Bact. Pasteurianum* at 26° to 30° C. consists of long chains, that of *Bact. Kützingianum*, on the contrary, of single cells, or pairs connected together. The same microscopical differences are thus found here as between the growths of both species on beer and wort gelatine.

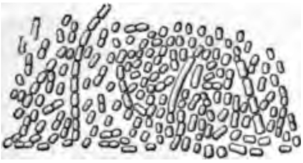


FIG. 145.—*Bacterium Kützingianum*, Hansen. Young film formation on "double" beer at 34° C. $\frac{1000}{1}$. (After Hansen.)

Of the many species of acetic acid bacteria which have been described in the last few years we will mention here among others :—

Bacterium oxydans, Henneberg (*Bact. aceti*, Zopf).—This species is distinguished from *Bact. aceti* of Hansen described above, by having, as shown by Zopf and Henneberg, a swarming state; it was isolated from a bottom fermented beer from Halle.

Further, Beijerinck mentions a species which he calls *Bact. aceti*, and which he states to be the active species in quick vinegar manufacture. It is distinguished by forming a film on artificial nutrient liquids; this property, held in common with other species, is, however, not permanent in this species, and may be lost after a previous cultivation on solid nutrient media. All gradations of film development are presented here. Further, it liquefies beer gelatine containing cane sugar, this being due, according to Beijerinck, to the inversion of the sugar.

To what extent Beijerinck's *Bact. aceti* is the acetic bacterium of quick vinegar manufacture is, however, not established. Rothenbach found that the active bacteria of the process named are not in a condition to form a film. The loss of this power of film formation rests, in his opinion, on the circumstance that they are never allowed the opportunity of film formation in practice, because the mash is in continual motion. According to Rothenbach the quick vinegar bacteria are acclimatised forms. Some of them closely resemble *Bact. aceti*, Hansen, and can furnish vinegar containing a high percentage of acetic acid from a mash containing little nutrient substance and a high percentage of alcohol, in the Schützenbach acetifiers.

Of other acetic acid bacteria the following may be mentioned :—

Bacterium Xylinum, A. J. Brown.—The gelatinous substance is cartilaginous, and furnishes the cellulose reaction with iodine and sulphuric acid, *i.e.*, a blue colour. This species also occurs in the acetifiers of the quick vinegar process. Further, the following is described by Henneberg :—

Bacterium acetigenum, which was isolated from the vinegar of a quick vinegar manufactory. It yields a cellulose reaction under certain conditions, and has especially intense power of swarming; **Bacterium industrium** also has a swarming state.

Several species have likewise been described under the generic name *Termobacterium*, as for example :—

Termobacterium aceti, Zeidler, which is illustrated in Fig. 135 and which possesses a long flagellum; further

Termobacterium lutescens, Lindner, which renders beer turbid and gives rise to a celery odour in it.

We will mention still a few more *Bacterium* species :—

Bacterium vermiforme, Marshall Ward, forms cells which measure 0·5 to 50 μ long and 0·5 μ broad. The gelatinisation of the cell wall with this species consists in the loosening of the strongly swollen outer layer of the cell membrane whereby the cells are enclosed as in a capsule. Together with the *Saccharomyces pyriformis* mentioned on p. 261 it forms the so-called ginger beer plant.

Bacterium termo, Cohn, is a collective expression for motile bacteria occurring in decomposing substances. According to Cohn's description it is a feebly fluorescent, strongly motile, short, ovate rod; but which of the numerous putrefactive bacteria is thus indicated cannot be determined.

Windisch considers that the cause of the so-called cellar flavour, by which is understood usually a raw, musty, damp taste in the beer, is undoubtedly to be looked for in the contamination of the wort during cooling, as the beer sometimes remains too long in the coolers during the warm summer months, and is, in consequence, infected by one of the bacteria belonging to the *Bact. termo* group.

They thrive well in hopped wort, but cannot live associated with yeast.

Most of the species included in the genus *Bacillus*, Cohn, form endospores. They can be classified in various groups according to their physiological activity.

Slime-forming Species.

Bacillus viscosus I. and II., van Laer, were isolated from ropy beers. Both form rods with a length of 1·6 to 2·4 μ and a breadth of 0·8 μ; they are usually isolated, not infrequently, however, in pairs. **I.** forms on the surface of wort slimy, yellowish islands which appear to branch downwards. They give rise, in consequence, to a slimy covering containing bubbles, which result from the evolution of carbonic acid. **II.** does not form a slimy covering. The carbonic acid production and viscosity are less. The wort becomes dark-brown and a peculiar odour is evolved. A solution of 3 grams of saccharose and 1 gram of peptone in 100 c.c. of water is rendered ropy and viscous by I., while II. only causes turbidity and carbonic acid evolution. A higher amount of sugar is prejudicial to the development of these bacteria, for which reason, therefore, lightly fermented beer is comparatively seldom ropy. The optimum temperature for their development is 33° C. The bacteria are frequently found in water analyses.

Bacillus viscosus III., L. Vandam, forms rods which are 2·0 μ long and 0·7 μ broad; they usually occur isolated; sometimes they are joined in chains of two or three members. This species was found in English beer; the ropiness is conditioned by the presence of sugar; air is necessary for the development of the species.

Bacillus viscosus vini, Kramer, makes white wine viscous in the absence of air. It forms rods 2 to 6 μ long and 0·6 to 0·8 μ broad: these are often joined in many-membered chains.

Lactic Acid-forming Species.

Mention has been already made of the discovery of lactic acid bacteria by Pasteur, of their importance in distilleries, and of their recent introduction into the latter in the form of pure cultures.

The first to isolate a pure lactic acid bacterium for the above purpose was, as has been said, F. Lafar. The species isolated and applied by him was **Bacillus acidificans longissimus, Lafar,** the cells of which are 2·5 to 25 μ long and about 1 μ broad.

Bacillus acidi lactici, Hueppe, consists of immotile rods which are 1·0 to 1·7 μ long and 0·3 to 0·4 μ broad; they are frequently connected in pairs, rarely in four-membered chains.

This bacillus is aërobic and does not liquefy gelatine. It loses its souring power if it is cultivated for a long time on a sugar-free nutrient medium. As a result of seeding this species in wine, Kramer produced "Zickenwerden" (lactic acid sharpness).

Saccharobacillus Pastorianus, van Laer, is responsible for that disease known as the "turning" of beer. Pasteur made the discovery that the disease is caused by bacteria. The species thrives best on malt extract-agar with a little alcohol added; this medium should be used when it is desired to obtain a pure culture. It develops in beer only when the latter contains a small amount of hop extract. According to van Laer it readily ferments saccharose (without inversion), maltose and dextrose, but lactose only with difficulty. Lactic acid, ethyl alcohol and small amounts of acetic and formic acid are found in the fermentation. Its development in beer is prevented by over 7 per cent. of alcohol.

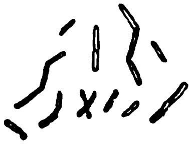


FIG. 146.—Lactic Acid Bacteria.

Butyric Acid-forming Species.

It has been stated that Pasteur discovered a butyric acid bacterium which he named *Vibrion butyrique*: later it was shown that this fermentation is caused by many species, which have already been alluded to as the cause of much harm in breweries.

Clostridium butyricum, Prazmowski (Bacillus amylobacter, van Tieghem), probably includes several species. The form described by Prazmowski (Figs. 134 and 137) consists of rods 1 μ broad, of which the young individuals contain a substance granulose which, however, is only formed if the water is rendered ropy and viscous by L., whil causes turbidity and carbonic acid evolution. amount of sugar is prejudicial to the developmen bacteria, for which reason, therefore, lightly ferm is comparatively seldom ropy. The optimum te for their development is 33° C. The bacteria are f found in water analyses.

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Bacillus acidi la [illegible] which are 1·0 to [illegible] are frequently c [illegible] chains.

This baci [illegible] It loses its [illegible] bacteria live in an entirely anaërobic state, and which, like starch (amylum), is stained blue by iodine. (From this the generic names *Amylobacter* and *Granulobacter*, sometimes used, are derived.) They are provided with flagella (Fig. 134), and display active motion. When the spores develop, the rods swell out at one end and become club-shaped (Clostridium form) (Fig. 137). According to Prazmowski, the spores survive the temperature of boiling water for five minutes; after ten minutes only a few of the strongest are alive, whilst all die in fifteen minutes. The species is strongly anaërobic.

Bacillus butyricus, Hueppe, differs from the above species in being aërobic.

Granulobacter saccharobutyricum, Beijerinck, is of general occurrence on barley corns, and therefore also on green malt, groats, flour, etc. It is one of the most injurious species in distilleries. It decomposes dextrose as well as maltose, and produces butyric acid, butyl alcohol, carbonic acid and hydrogen; gelatine is not liquefied by this species.

Bacillus lupuliperda, Behrens, is classified here inasmuch as it forms butyric acid when it is cultivated in a nutrient liquid containing sugar. Behrens found it in hops which had become warm. The cells are motile; they are 0·7 μ to 2·5 μ long and 0·7 μ broad. It liquefies gelatine, and can be cultivated in an extract of hops. This bacillus is partly the cause of the spontaneous heating of hops, and forms trimethylamine and ammonia.

Finally, two bacilli have yet to be mentioned which cannot be classified in the above groups. One is

Bacillus piluliformans, Müller-Thurgau.—The rods are 3 μ to 10·5 μ long, most frequently 4 μ to 6 μ, and are 0·75 μ broad. It causes in wine a remarkable disease described by Müller-Thurgau. In a red wine it had formed rounded corn-like grains, which clung to the sides of the bottle, but mostly to the bottom; these were in greater part detached by gently moving the bottle. The grains, of which as many as a hundred were found in a bottle, varied very much in size, some being hardly discernible, whilst others had a diameter of as much as 4·5 mm. The wine was of a darker colour than usual; its flavour and smell were but slightly altered; it was clear, but not of good quality.

Bacillus subtilis, Ehrenberg. Hay bacillus.—It is found frequently on hay. The spores are very resistant towards high temperatures; according to Brefeld, they survive heating to 100° C. for three hours, to 105° C. for a quarter of an hour, and to 110° C. for five minutes. This species can therefore be obtained by pouring water over hay and boiling the liquor drawn off. The rods are strongly motile, and are furnished with several flagella. It first inverts cane sugar, and then oxidises it to the last trace. During its action on dextrose a strongly reducing lævo-rotatory body is formed (A. J. Brown).

This species is frequently met with in physiological fermentation work. It develops easily in unhopped wort; it does not thrive in acid liquids, and is on that account without significance in the brewery. According to Esaulow, it occurs in kefir; it here participates in the formation of kefir grains, the film it forms on the milk serving as a collecting place for the lactic acid bacteria and yeast.

LITERATURE REVIEW.

SECTION I. (Pages 1—15).

I.—**SPALLANZANI, LAZZARO**: Saggio di osservazioni microscopiche, relative al sistema della generazione di signori Needham e Buffon. Modena, 1765.

II.—**SCHEELE, CARL WILHELM**: Anmärkningar om sättet att conservera ättika. (Kongl. Vetenskaps Academiens nya Handlingar. Tom. iii. Stockholm, 1782, p. 120.)

III.—**APPERT**: Le livre de tous les ménages ou l'art de conserver pendant plusieurs années, toutes les substances animales et végétales. 4ième éd. Paris, 1831.

IV.—**SCHULZE, FRANZ**: Vorläufige Mittheilung der Resultate einer experimentellen Beobachtung über generatio æquivoca. (Poggendorff's Annal. d. Phys. u. Chem. Bd. xxxix., 1836. No. 11, p. 487.)

V.—**CAGNIARD LATOUR, CHARLES**: (1) His communication appeared in the Parisian "L'Institut" on 23rd November, 1836.

——— (2) Mémoire sur la fermentation vineuse. (Laid before the Académie des Sciences on 12th June, 1837; published in Compt. rend. de l'Acad. des Sc., Tom. iv., 1837, p. 905, and in Annal. de Chim. et Phys., Tom. lxviii., 1838, p. 206.)

Cagniard Latour states in the above that yeast is organised matter and that it is probable that the formation of carbonic acid and of alcohol is caused in some way by the growth of the yeast.

VI.—**SCHWANN, THEODOR.**

In September, 1836, Schwann demonstrated before the Naturforscher-Versammlung in Jena that the yeast fungus described by him is the cause of alcoholic fermentation.

In the publication which appeared in 1837 under the following title—

——— (1) Vorläufige Mittheilung, betreffend Versuche über die Weingärung und Fäulnis (Poggendorff's Annal. d. Phys. u. Chem. Bd. xli., 1837, Nr. 5)—

he also communicates the discovery that putrefaction is caused by living corpuscles. His words are:—

". . . Then putrefaction must be thus explained, that these germs, while developing and while feeding at the expense of the organic substance, cause in it a decomposition of such a nature that it gives rise to the phenomena of putrefaction. . . ."

——— (2) Mikroskopische Untersuchungen über die Übereinstimmung in der Struktur und dem Wachsthum der Thiere und Pflanzen. Berlin, 1839.

In the above research, Schwann again establishes that yeast is a fungus, and that it causes fermentation. He further communicates his discovery of yeast spores and lays the foundation of the science of antiseptics. He expresses himself thus:—

". . . All conceivable proofs point to the ferment particles being fungi. Their form is that of the fungi, their structure is like that of the fungi, for they consist of cells many of which again contain young cells; they grow like fungi by producing new cells at their ends, they propagate themselves like fungi, partly by separation of single cells, partly by production of new cells in the existing ones, and by the bursting of these mother cells. Now, that these fungi are the cause of fermentation follows: first, because they constantly occur in fermentation; second, because fermentation is stopped by all processes which evidently kill the fungi, *e.g.*, boiling heat, potassium arsenate, etc. . . ."

VII.—KÜTZING, FRIEDRICH: Mikroscopische Untersuchungen über die Hefe und Essigmutter, nebst mehreren anderen dazu gehörigen vegetabilischen Gebilden. (Journ. f. prakt. Chemie, Jahrg. 1837, Bd. ii., p. 385.)

VIII.—v. LIEBIG, JUSTUS: (1) Die organische Chemie in ihrer Anwendung auf Agrikultur und Physiologie. 1. Aufl., 2. Theil. Braunschweig, 1840.

Liebig had as early as 1839 expressed the leading principles of his theory in a treatise: "Über die Erscheinungen der Gährung, Fäulnis und Verwesung und ihre Ursachen". (Poggendorff's Annal. d. Phys. u. Chem., 1839, pp. 106-150.) He expresses the following views on p. 142: "The form and condition of the insoluble precipitates in fermentation have led to a very strange conception of fermentation on the part of many physiologists. When beer and wine yeasts are distributed in water and looked at under a good magnifying glass, transparent, flat, compressed corpuscles are seen, which sometimes, attached to one another in chains, assume the form of growths; in the eyes of others they are similar to many infusoria. It would certainly be an extremely noteworthy phenomenon if plant substance and albumen, which are precipitated in a changed condition in the fermentation of beer and of plant juices, were to assume a geometrical form when deposited, since these bodies have never been observed in the crystallised condition. Now this is not the case; they precipitate, like all substances which do not possess crystalline properties, in the form of spherical corpuscles, which either swim about free or are connected with others. These investigators were misled by this form to regard the ferment as consisting of animated organic beings, whether plants or animals, which in their development assimilate the constituents of sugar and excrete them again in the form of carbonic acid and alcohol; they explain the decomposition of the sugar and the increase of the mass of the added ferment in beer fermentation. This view confutes itself; in pure sugar water the so-called seeds disappear with the plants on fermentation; fermentation takes place, the decomposition of the sugar ensues with that of the ferment, without any development or reproduction of seeds, plants or animals being observed, which is regarded by these investigators as the cause of the chemical process."

Liebig, Justus: (2) Ueber die Gährung und die Quelle der Muskelkraft. (Sitzungsber. der Königl. bayer. Akad. d. Wissensch. ii., 1869, pp. 323 and 393, and Annal. d. Chemie u. Pharmacie, Bd. cliii., 1870, pp. 1 and 137.)

In the above treatise, Liebig's last opinion on the Pasteur fermentation theory is expressed. It may be inferred from the following two quotations:—

Page 2: "From the chemical standpoint, which I should not like to give up, it is an 'act of life,' 'a condition of motion,' and taken in this sense Pasteur's view is not opposed to mine nor does it disprove mine".

Page 6: "It might be that the physiological process stands in no other relation to the process of fermentation than to produce the substance in the living cell, which, by an action peculiar to itself similar to that of emulsin on salicin and amygdalin, causes the breaking up of the sugar and other organic atoms; the physiological process would be necessary in this case to produce the above substance, but with the fermentation as such it would have no further connection ".

Hoppe-Seyler in his "Physiologische Chemie," Berlin, 1877, expresses substantially the same view as that of Liebig's given above and that given formerly by Traube. According to Hoppe-Seyler, fermentation is caused by a purely chemical ferment contained by the yeast. On page 115 he says: "Liebig has shown very clearly the untenable and harmful nature of Pasteur's views, yet his deductions received little attention ".

IX.—MITSCHERLICH, EILHARD: (1) Über die chemische Zersetzung und Verbindung vermittelst Contactsubstanzen, given as a lecture in the Kgl. Akad. d. Wissensch. Berlin; a reference is to be found in Bericht über die zur Bekanntmachung geeigneten Verhandlungen d. Kgl. preuss. Akad. d. Wissensch. zu Berlin, Dec., 1841.

On p. 390 he writes as follows: "The sugar into which cane sugar changes, when yeast is added to a solution of the latter, appears to be different to grape sugar; the author has not been able to crystallise it, and it polarises light much less than the same quantity of grape sugar; its formation is very remarkable; it is in fact a substance mixed with the yeast spherules, and can be removed by water extraction, and a clear solution of it causes the transformation of cane sugar into this kind of sugar ".

——— (2) Another communication is to be found *ibid.*, Feb., 1843.

He exhibited on this occasion some engravings which represented among other things the increase of top yeast. The figure given on p. 192 of my book is a reproduction of one of these engravings, which, however, so far as can be found, were never published all together. Some of the figures reproduced are to be found in Mitscherlich's "Lehrbuch der Chemie," 4. Ausg., Bd. i., 1844; and in Regnault, "Cours élémentaire de chimie," 2me éd., tom. iv., p. 179; further in Jos. Bersch, "Gährungs-Chemie für Praktiker," 1. Teil, Berlin, 1879, p. 60, etc.

X.—SCHROEDER, H., und VON DUSCH, TH.: Ueber Filtration der Luft in Beziehung auf Fäulnis und Gährung. (Annal. der Chem. und Pharm. Bd. lxxxix., Heft 2, 1854, p. 232.)

XI.—PASTEUR, LOUIS: (1) Mémoire sur la fermentation appelée lactique. (Compt. rend. de l'Acad. des Sc., Tom. xlv., 1857, p. 913.)

The above memoir is Pasteur's first research on this subject, and treats, as the title shows, of lactic acid fermentation. In the same year he published his first paper on alcoholic fermentation, *viz.*:—

ASTEUR, LOUIS: (2) Mémoire sur la fermentation alcoolique. (*Ibid.*, p. 1032.)

He arrives at the same results here as his predecessors, Schwann, etc., *viz.*, that yeast splits up the sugar in consequence of its vitality.

He then publishes a series of smaller papers in the "Compt. rend."; all matter referring to alcoholic fermentation is collected in a larger paper, *viz.*:—

——— (3) Mémoire sur la fermentation alcoolique. (Ann. de Chim. et de Phys. 3 Sér., Tom. xlviii., 1860, p. 323.)

The publications in the "Compt. rend." are continued, and among these might be mentioned:—

——— (4) De l'origine des ferments. Nouvelles expériences relatives aux générations dites spontanées. (Compt. rend. de l'Acad. des Sc., Tom. l., 1860, p. 849.)

in which he controverts the doctrine of generatio aequivoca, and:—

——— (5) Fermentation butyrique. Animalcules infusoires vivant sans oxygène libre et déterminant des fermentations. (Compt. rend. de l'Acad. des Sc., Tom. lii., 1861.)

in which, for the first time, the doctrine of anaërobiosis is put forward.

For some years he continues to fight against the doctrine of generatio æquivoca; his particular opponents on this point are Pouchet and Joly; after him his pupils, especially Chamberland and Roux, take up the matter.

Pasteur at the same time upholds his theory of fermentation till the beginning of the seventies; his writings in this connection are to be found in the "Compt. rend." His opponents are chiefly Liebig, Traube, Brefeld, Berthelot and Cl. Bernard.

——— (6) Études sur le vin. Paris, 1866.

——— (7) Études sur le vinaigre. Paris, 1868.

——— (8) Études sur la bière. Paris, 1876.

In the three researches named, which with (4) must be looked upon as the chief works of Pasteur in the science of fermentation, is to be found what he has accomplished for these branches of industry; in (8), especially, there is a collation of his researches on fermentation and the organisms of fermentation as a whole, and the book practically ends his researches on this subject. He publishes here for the first time his methods for purifying yeast (the tartaric acid method, etc.), which were kept secret in his first smaller communications.

While the works of Pasteur on generatio æquivoca and on his fermentation theory caused prolonged contests, his attempts to introduce reform into technical fermentation very soon came to an end. His methods and suggestions were tested, but without giving successful results, as the most important thing, the pure yeast, was wanting. In Denmark, the Messrs. Jacobsen began experiments in the Old and New Carlsberg breweries shortly after Pasteur's work had appeared in 1876, using the methods there recommended for the purification of the yeast; but the results were not satisfactory. This was the case also in France and other countries. His "Études sur la bière" are mentioned in the technical writings of that time with respect, but gradually become treated as something which had little to do with practice; they did not come into vital contact with the latter. The only upholder of Pasteur's work on technical fermentation is Velten, his co-worker on the subject of brewing. In the "Revue universelle de la brasserie et malterie," 1888, No. 742 and No. 743, he points out that it is the mixture of several different pure species of yeast which gives the beer the required flavour and bouquet. This is obtained, he asserts, according to Pasteur, by cultivation of the yeast in a cane sugar solution to which a little tartaric acid has been added, or in wort to which carbolic acid and alcohol have been added. As early as 1878 he had given, in the lectures which he delivered to the Congress at the International Exhibition in Paris, an exact description of this method, as Pasteur and he considered that the brewers should use it for the purification of their yeast. Velten published these lectures later under the title, "De la fabrication de la bière par le procédé Pasteur" (Revue universelle de la brasserie, 1881, No. 372). They are also to be found in the publications of the Congress.

Duclaux also recommends Pasteur's above named methods in his "Chimie biologique," 1883, p. 301. As is mentioned in the introductory chapter, no one understood at that time the importance of the disease yeasts and their competition with the culture yeast; consequently also, it was unknown that Pasteur's method was specially favourable to the development of yeast diseases in beer. Thus in the above work Duclaux, in agreement with Pasteur, mentions (p. 618) only bacteria as the disease germs of beer. This, however, did not prevent him and other members of the French school from attempting later on to give quite a different interpretation to "Études sur la bière". It became a regular practice to find in this work the new results produced by science after 1876 on the biology of yeast fungi and their use in technique. "Études sur la bière" abounds in contradictory opinions where the yeast fungus is treated, without being decisive as to which is the right one. This is the case, *e.g.*, in the question of the parent forms of yeast species (pages 155, 165, 177), and on top yeast and bottom yeast. (On p. 189 of the work cited, the view is expressed that a conversion of the one into the other does not take place, but on p. 338 the contrary is stated, *viz.*, that the conversion of bottom yeast into top yeast can take place in breweries, a method even being described to prevent this.) By this means much confusion in the literature, and now and then little disputes, have been caused. The discussions of possibilities which occur in so many places in Pasteur's work enabled any one to find nearly always what he desired. "Études sur la bière" was not used as a scientific work, but rather as a Bible.

On the dispute which Hansen's work caused see p. 355.

XII.—**Traube, Moritz**: Theorie der Fermentwirkungen. Berlin, 1858.

XIII.—**de Seynes, Jules**: Sur le Mycoderma vini. (Compt. rend., Tom. lxvii., 1868, p. 105.)

XIV.—**Reess, Max**: Botanische Untersuchungen über die Alkoholgährungspilze. Leipzig, 1870.

XV.—**Brefeld, Oscar**: (1) Methoden zur Untersuchung der Pilze. (Ber. d. med.-phys. Ges. zu Würzburg, 1874; also in Landwirtsch. Jahrb., Bd. iv., 1875, p. 160.)

——— (2) Botanische Untersuchungen über Schimmelpilze. Heft 2 und 4, 1874 and 1881.

XVI.—**Lister, Joseph**: On the Lactic Fermentation and its bearing on Pathology. (Trans. of the Path. Soc. of London, Vol. xxix., 1878, p. 445.)

XVII.—**Holzner, Georg**: Zymotechnische Rückblicke auf das Jahr 1877. (Zeitschr. f. d. ges. Brauw., 1878, p. 142.)

XVIII.—**v. Nägeli, Carl**: Theorie der Gärung. München, 1879.

XIX.—**Hansen, Emil Chr.**: (1) Contributions à la connaissance des organismes qui peuvent se trouver dans la bière et le moût de bière et y vivre. (Compt. rend. des trav. du laborat. de Carlsberg, Tom. i., livr. ii., 1879, p. 49.)

In the above work Hansen communicates the preliminary investigations for his reforming researches published in the following years.

Hansen, Emil Chr.: (2) Recherches sur les organismes qui, à différentes époques de l'année, se trouvent dans l'air, à Carlsberg et aux alentours, et qui peuvent se développer dans le moût de bière. (*Ibid.*, Tom. i., livr. iv., 1882.)

In the note on p. 206 the chief features of Hansen's spore analysis are given. His first pure culture method is described on p. 212, and on p. 217 are described the first experiments on diseases in beer caused by fungi of alcoholic fermentation.

——— (3) Maladies provoquées dans la bière par des ferments alcooliques. (*Ibid.*, Tom. ii., livr. ii., 1883, p. 52.)

——— (4) Les ascospores chez le genre Saccharomyces. Méthodes. (*Ibid.*, Tom. ii., livr. ii., 1883, p. 28.)

——— (5) Méthodes pour obtenir des cultures pures de Saccharomyces et de microorganismes analogues. (*Ibid.*, Tom. ii., livr. iv., 1886, p. 92.)

——— (6) Qu'est-ce que la levûre pure de M. Pasteur? (*Ibid.*, tom. iii., livr. i., 1891, p. 24.)

The most important researches of Hansen are contained in the four works mentioned below.

——— (7) Recherches sur les organismes qui, à différentes époques de l'année, se trouvent dans l'air, à Carlsberg et aux alentours, et qui peuvent se développer dans le moût de bière. (Compt. rend. des trav. du laborat. de Carlsberg, 1879, 1882.)

——— (8) Recherches sur la physiologie et la morphologie des ferments alcooliques. (*Ibid.*, 1881, 1883, 1886, 1888, 1891, 1898, 1900, 1902.)

——— (9) Recherches sur les bactéries acétifiantes. (Compt. rend. des trav. du laborat. de Carlsberg, 1879, 1894, 1900.)

——— (10) Untersuchungen aus der Praxis der Gärungsindustrie.

The first edition of this work is given in Danish, with a *résumé* in French in the "Compt rend. des trav. du laborat. de Carlsberg," 1888, 1892. Of the more complete German edition (Oldenbourg,

Munich and Leipzig) there appeared: Part I., edition 1, 1888; edition 2, 1890; edition 3, 1895; Part II., 1892. The collected English edition appeared in 1896. (Spon, London and New York.)

The first to advocate Hansen's pure culture system in the literature was Carl Lintner, sen., who was then professor and director of the Royal Bavarian Agricultural Centralschule in Weihenstephan (Zeitschr. f. d. ges. Brauw., 1884, p. 245). Aubry at the same time introduced Hansen's innovations into the scientific brewing station at Munich. In his report for the year 1897 (p. 591 in the above journal), Will mentions that "the epoch-making ideas of Hansen have found a home and fostering ground in this station for more than a decade". "Our station," he adds, "was the only one for a long time which disseminated them among brewing circles on the Continent and gave them due recognition. The stormy period and the vigorous struggle is probably still in the memory of each of us, which was necessary to overcome the many prejudices which opposed the introduction of the pure cultivated yeast into practice, not only on the part of practical men, but also of eminent theorists, and to allay the misunderstandings which were always cropping up." Later on the acceptance became general.

In Denmark, Chr. Grönlund and Alfr. Jörgensen in particular advocated Hansen's reforming works, and the latter particularly has striven to obtain their general acceptance. (See his text-book "Die Microorganismen der Gärungsindustrie," 1-4 editions, 1886-1898; also "Centralbl. f. Bakt., Parasitenk. u. Infektionskr.," 2 Abt., 1897, p. 665, and finally the section: "Die Theorie der Gärung," in Thausing's "Malzbereitung und Bierfabrikation," 1898, p. 651, besides his numerous articles in the brewing journals.) In this connection may be named also the two jubilee reports which the Old and New Carlsberg breweries published in 1896 and 1897 respectively; likewise the articles published by Kjeldahl in the "Nationaltidende," 15th May, 1887, and 10th November, 1897, by W. Johannsen in his textbook and E. Rostrup in the "Biographisk Lexikon".

Particulars of the dispute which the pure culture system aroused are given in the "Wochenschr. f. Brauerei" and the "Zeitschr. f. ges. Brauw.," in the years following 1883. Velten and Duclaux have been mentioned on p. 352. The attacks were not always made in the most considerate manner, and they have been continued down to the present time. (On this point see J. Chr. Holm, "Hansen's Reinzuchtsystem in Frankreich, Centralbl. f. Bakt., Parasitenk. u. Infektionskr.," 2 Abt., 1899, p. 641, where still further references are given; they supplement the final chapter in Jörgensen's text-book, 1898.)

In the introduction to his "Mikroskopische Betriebskontrolle in den Gärungsgewerben," 1 Aufl., 1895; 2 Aufl., 1898, P. Lindner on

p. 10 writes appreciatively: "Hansen by the use of the pure culture becomes the reformer in the science of yeast organisms, as Koch was in that of bacteria". The object of his book, however, consists in substituting in several places other methods in place of those introduced by Hansen; methods, *viz.*, which more resemble those of Koch. In it, he attacks several points in Hansen's work in that direction which have been defended by Alfr. Jörgensen and Will. The latter says (Zeitschr. f. d. ges. Brauw., 1899, p. 612): "The pioneer researches of Hansen have brought about a complete revolution in this direction. The extension of Pasteur's study of the organisms which produce diseases in beer, by Hansen, who showed that diseases are also produced by yeast species, and the building up of this theory by the latter investigator, his pupils and the zymo-technical laboratories, have advanced to such a point, that to-day we can with comparative quickness and certainty, by characteristic appearances, recognise all those organisms, in particular those yeasts, which are foreign to a normal yeast or fermentation and consequently also to a normal beer." Will and Jörgensen themselves have contributed. Among others, Prior's laboratory also took up Hansen's analytical methods. The latter depend in general on the study of important and characteristic physiological functions, but morphological characteristics were also included where this was possible. The same analysis can, of course, in many cases be performed in different ways. There is room enough for other methods besides those given by Hansen.

For illustrating the progress of the development it is instructive to go through various successive editions of textbooks and handbooks of the fermentation industries, *e.g.*, such works as that of Thausing mentioned above. Even at the beginning of the eighties no mention is made of a reform with regard to the yeast question; but in the following years Hansen's ideas make themselves increasingly felt.

As regards the pure culture system in wine manufacture, see Wortmann's book, "Die Anwendung und Wirkung reiner Hefen in der Weinbereitung," Berlin, 1895. On the pure culture system in spirit and pressed yeast manufacture, see Delbrück and P. Lindner in the "Zeitschr. f. Spiritusindustrie," and "Wochenschr. f. Brauerei," 1892 and 1895.

Although a large part of Hansen's investigations deals with the study of species, and although they depart from new points of view and have given rise to important progress, systematic botany has hitherto paid little or no attention to them. Descriptive botanists repeated substantially the methods of description of Reess, and proceeded from the old fallacy that a microscopical investigation is sufficient to distinguish the systematic units from one another. It is still more unfortunate that such a doctrine is taught in more than one of the Technische Hochschulen. The theoretical aspects of Hansen's research received special recognition in mycological textbooks (*e.g.*, Zopf's Die Pilze, 1890), and in those of the chemistry of fermentation (*e.g.*, Bourquelot, 1889; Langer, 1890; Ad. Mayer, 1895; Prior, 1896). It has introduced a new chapter into this literature.

XX.—LINTNER, CARL: Über Malz und dessen Einfluss auf die Haltbarkeit und Güte des Bieres. (Zeitschr. f. d. ges. Brauw., 1880, p. 384.)

XXI.—KOCH, ROBERT: (1) Zur Untersuchung von pathogenen Organismen. (Mitt. aus d. Kaiserl. Gesundheitsamte, Bd. i., 1881, p. 1.)

——— (2) According to Hueppe, the important plate culture was first demonstrated on the occasion of the Hygiene Exhibition in a lecture given by Koch during the XI deutschen Ärztetage, 1883, at Berlin. Special directions for these cultures were first published by Biedert in the Deutsche Medicinal-Zeitung, 1884.

XXII.—THAUSING, JULIUS: Einfluss der Hefegabe auf Hauptgährung, Hefe und Bier. (Allg. Zeitschr. f. Bierbrauerei, 1884, p. 872.)

XXIII.—FISCHER, EMIL: (1) Die Chemie der Kohlenhydrate und ihre Bedeutung für die Physiologie. Berlin, 1894.

——— (2) Einfluss der Konfiguration auf die Wirkung der Enzyme. (Ber. d. deutsch. chem. Ges., Bd. xxvii., 1894, and xxviii., 1895.)

XXIV.—DELBRÜCK, MAX: Lecture. (Wochenschr. f. Brauerei, Bd. xii., 1895, No. 30, pp. 732-733.)

XXV.—BUCHNER, EDUARD: Fortschritte in der Chemie der Gärung. Tübingen, 1897.

SECTION II. (Pages 16 to 169).

1. *Text-Books, Handbooks and Journals.*

DE BARY, A., and WORONIN, M.: Beiträge zur Morphologie und Physiologie der Pilze. 2. Reihe. Frankfurt, 1866.

BEHRENS, W.: Tabellen zum Gebrauch bei mikroskoipschen Arbeiten. Braunschweig, 1898.

DIPPEL, L.: Das Mikroskop und seine Anwendung. Braunschweig, 1882.

FRIEDLÄNDER: Mikroskopische Technik. Bearb. v. Eberth. 6. Aufl. Berlin, 1900.

HUEPPE, F.: Die Methoden der Bakterienforschung. 5. Aufl. Wiesbaden, 1891.

JÖRGENSEN, A.: Die Mikroorganismen der Gärungsindustrie. 4. Aufl. Berlin, 1898.

LAFAR, F.: Technische Mykologie. I. Schizomyceten-Gärungen. Jena, 1897.

LINDNER, P.: Mikroskopische Betriebskontrolle in den Gärungsgewerben. Berlin. 1 Ausg., 1895; 2 Ausg., 1898.

LINTNER, C. J.: Handbuch der landwirtschaftl. Gewerbe. Berlin, 1893.

Considers the application of the pure culture system not only in breweries, but also in distilleries and in pressed yeast manufacture.

MORITZ and MORRIS: A Text-book of the Science of Brewing. London, 1891.

STRASBURGER, E.: Das botanische Practicum. Jena, 1887.

SYKES, W. J.: The Principles and Practice of Brewing. London, 1897.

References to the pure yeast system in English top fermentation breweries.

Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten. 2. Abt. Herausg. von Oscar Uhlworm and Emil Chr. Hansen. Jena, 1895 f.

Jahresbericht über die Fortschritte in der Lehre von den Gärungsorganismen. Herausg. von Alfr. Koch. Braunschweig, 1891.

Zeitschrift für wissenschaftliche Mikroskopie. Herausg. von W. J. Behrens. Braunschweig, 1884 f.

2. *Special Works.*

Brefeld, O.: Methoden zur Untersuchung der Pilze. (Ber. d. med.-phys. Ges. zu Würzburg, 1874; also in Landwirtsch. Jahrb. Bd. iv., 1875.)

——— Botanische Untersuchungen über Schimmelpilze. Parts 2 and 4, 1874 and 1881.

The above researches are specially important as regards methods of studying morphology and life history.

Greg, P. H.: Fermentation in Rum Distilleries. (The Sugar-Cane. Vol. xxv., 1893, No. 292.)

Hansen, Emil Chr.: Contributions à la connaissance des organismes qui peuvent se trouver dans la bière et le moût de bière et y vivre. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. i., livr. ii., 1879, p. 49.)

——— Sur le Saccharomyces apiculatus et sa circulation dans la nature. (*Ibid.*, Tom. i., livr. iii., 1881, p. 159.)

——— Recherches sur les organismes qui, à différentes époques de l'année, se trouvent dans l'air, à Carlsberg et aux alentours, et qui peuvent se développer dans le moût de bière. (*Ibid.*, Tom. i., livr. iv., 1882, p. 197.)

Here also are found the principles of Hansen's spore analysis (p. 206) and his first pure culture method (p. 212).

——— Les ascopores chez le genre Saccharomyces. (*Ibid.*, Tom. ii., livr. ii., 1883, p. 13.)

On p. 23 and following there is a somewhat more detailed description of Hansen's pure culture methods and of his spore culture method.

——— Über Wiesner's neue Prüfungsmethode der Presshefe. (Dingler's polytechn. Journal, 1884.)

——— Méthodes pour obtenir des cultures pures de Saccharomyces et de microorganismes analogues. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. ii., livr. iv., 1886, p. 92.)

A detailed description of the manipulations in his pure culture methods.

HANSEN, EMIL CHR: Les voiles chez le genre Saccharomyces. (*Ibid.*, Tom. ii., livr. iv., 1886, p. 106.)

——— Untersuchungen aus der Praxis der Gärungsindustrie, Heft 1, 1. Ausg., 1888; 3. Ausg., 1895, Heft 2, 1892. München und Leipzig.

The contents of Part 1 are as follows: 1. The pure culture of methodically selected yeasts in the service of the industry. 2. Studies of yeast species. 3. On the practical testing of beer in lager casks, with respect to its stability. Part 2 contains: 1. The technical analysis of the micro-organisms of air and water. 2. What is Pasteur's pure yeast? 3. Investigations on diseases caused in beer by alcoholic fermentation fungi. 4. On the present-day use of my pure yeast culture system.

The majority of the above works are to be found in Danish, with *résumés* in French, in the journal of the Carlsberg laboratory. Also the English edition mentioned above, London, 1896.

——— Sur la vitalité des ferments alcooliques et leur variation dans les milieux nutritifs et à l'état sec. (*Ibid.*, Tom. iv., livr. iii., 1898, p. 93.)

Methods are given here for preserving pure not only yeast species, but also alcoholic fermentation fungi in general.

——— Neue Untersuchungen über die Sporenbildung bei den Saccharomyceten. (Centralbl. f. Bakt., Par. u. Inf. 2. Abt., Bd. v., 1899, No. 1.)

——— La variation des Saccharomyces. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. v., livr. i., 1900.)

HOLM, J. CHR.: Sur les méthodes de culture pure et, spécialement, sur la culture sur plaques de M. Koch et la limite des erreurs de cette méthode. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. iii., livr. i., 1891, p. 1. In German in Zeitschr. f. d. ges. Brauw., 1891, S. 458.)

——— Analyses biologiques et zymotechniques de l'eau destinée aux brasseries. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. iii., livr. 2, 1892, p. 107.)

HOLM, J. CHR., et POULSEN, S. V.: Jusqu'à quelle limite peut-on, par la méthode de M. Hansen, constater une infection de "levûre sauvage" dans une masse de levûre basse de Saccharomyces cerevisiæ? (Compt. rend. des travaux du laborat.

de Carlsberg, Tom. ii., livr. iv., 1886, p. 88 et Tom. ii., livr. v., 1888, p. 137.)

Jörgensen, Alfr.: Vorläufiger Bericht über Versuche im grossen mit absolut reiner Oberhefe, nach Hansen's Methode dargestellt. (Allg. Zeitschr. f. Bierbr. u. Malzfabr., 1885, No. 36.)

This has a special interest as it is the first communication on the application of pure yeast species in top fermentation.

——— Die Anwendung der nach Hansen's Methode reingezüchteten obergärigen Hefe in der Praxis. (Brauer- u. Mälzer-Kalender f. Deutschland u. Österreich. Stuttgart, 1889-90.) Referat von Will in Zeitschr. f. d. ges. Brauw., 1890.

It is shown here that in certain cases a special aëration of the wort can hasten the clearing.

——— Zur Analyse der obergärigen Hefe in Brauereien und Brennereien nach Hansen's Methode. (Zeitschr. f. d. ges. Brauw., 1891.)

——— Über die Anwendung von Hansen's System in Obergärungsbrauereien. (*Ibid.*, 1893.)

——— On Hansen's System of Pure Yeast Culture in English Top Fermentation. (Trans. of the Inst. of Brewing. Vol. vii., 1894.)

In the above two communications the same subject is treated but from different standpoints. A special interest attaches to the communication of 1893.

Jörgensen, Alfr., et Holm, J. Chr.: Le procédé de M. Effront pour la purification et la conservation de la levûre à l'acide fluorhydrique et des fluorures. (Monit. scient. du Dr. Quesneville. 4 Sér. Tom. vii., 1893. Zeitschr. f. d. ges. Brauw., 1893.)

Koch, R.: Zur Untersuchung von pathogenen Organismen. (Mitt. aus dem Kaiserl. Gesundheitsamte, Bd. i., Berlin, 1881.)

Fundamental investigations for the technique as regards the use of nutrient gelatine for cultivating micro-organisms.

Miquel: Dixième mémoire sur les poussières organisées de l'air et des eaux. (Annuaire de Montsouris. Paris, 1888.)

Of importance as regards the methods of examining air and water.

MÜLLER-THURGAU, H.: Neue Forschungsresultate auf dem Gebiete der Weingärung und deren Bedeutung für die Praxis. (Ber. über d. Verh. d. xi. deutsch. Weinbau-Kongresses in Trier, 1889.)

——— Ergebnisse neuer Untersuchungen auf dem Gebiete der Weinbereitung. (Ber. über d. Verh. d. xii. deutsch. Weinbau-Kongresses, 1891.)

——— Über neuere Erfahrungen bei Anwendung der Reinhefen in der Weinbereitung. Mainz, 1896.

PASTEUR, L.: Études sur la bière. Paris, 1876.

Of the extremely rich and varied contents the following are specially important for this section: Descriptions of various culture flasks (pp. 27, 29, 88, 328 and 331), sterilisation of nutrient solutions (pp. 29 and 332), composition of artificial nutrient liquids (pp. 89 and 319), etc.

SCHIÖNNING, H.: Matras pour cultures sur blocs de plâtre. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. iv., livr. ii., 1896, p. 89.)

SEIFERT, W.: Verwendung von Reinzuchthefen in der Kellerwirtschaft. (Österr. landwirtsch. Wochenbl., 1897.)

WICHMANN, H.: Neuere Hefereinzucht-Apparate. (Mitt. d. österr. Vers.-Stat. f. Brauerei u. Mälzerei in Wien, Heft 6, 1894.)

WILL, H.: Wie wird reine Hefe gezüchtet? (Zeitschrift f. d. ges. Brauw., 1885.)

——— Notiz betr. den Nachweis von wilden Hefearten in Brauereihefen und Jungbieren, sowie das Vorkommen von Sacch. apiculatus in denselben. (*Ibid.*, 1893.)

——— Zur Untersuchung hefentrüber Biere. (Forschungsber. über Lebensmittel. 1. Jahrg., Heft 10, 1894.)

——— Die Methoden, welche bei der Reinzüchtung von Hefe und ähnlichen Organismen durch Einzellkultur auf festem Nährboden zur Feststellung der Lage der ausgewählten Zellen in den Kulturen zur Anwendung kommen. (Centralbl. f. Bakt. u. Par., 2 Abt., Bd. ii., 1896.)

WILL, H.: Einige Bemerkungen über die Lebensdauer getrockneter Hefe. (Zeitschr. f. d. ges. Brauw., 1896. Addenda are found *ibid.*, 1897, 1898 and 1899.)

——— Einiges aus der Praxis des physiologischen Laboratoriums. (Zeitschr. f. d. ges. Brauw., 1899.)

WORTMANN, J.: Untersuchungen über reine Hefen i., ii. (Landwirtsch. Jahrb. 1892 und 1894.)

——— Über die Verwendung von reinen Weinhefen bei der Apfelweinbereitung. (Weinbau und Weinhandel, 1893.)

——— Über die Anwendung von rein gezüchteten Hefen bei der Schaumweinbereitung. (*Ibid.*)

——— Mitteilung über die Verwendung von konzentriertem Most für Pilzkulturen. (Botan. Zeit., Bd. li., 1893.)

——— Die Verwendung und Bedeutung reiner Hefen bei der Weinbereitung. (Ber. über die Verh. d. deutsch. Weinbauvereins in Neuenahr, 1893.)

——— Anwendung und Wirkung reiner Hefen in der Weinbereitung. Berlin, 1895.

Comprehensive review and guide for practical men.

——— Über Fehler, welche bei Anwendung von Reinhefen gemacht wurden. (Ber. über d. Verhandl. des xvii. deutschen Weinbau-Kongresses in Trier, 1898.)

——— Untersuchungen über das Umschlagen der Weine. (Weinbau und Weinhandel, 1899.)

SECTION III. (Pages 170 to 345).

1. *Text-Books, Handbooks and Journals.*

DE BARY, A.: Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bacterien. Leipzig, 1884.

DUCLAUX, E.: Chimie biologique. Paris, 1883.

JÖRGENSEN, ALFR.: Die Mikroorganismen der Gärungsindustrie. 4. Aufl. Berlin, 1898.

LINDNER, P.: Mikroskopische Betriebskontrolle in den Gärungsgewerben. 2. Aufl. Berlin, 1898.

MAYER, ADOLF: Die Gärungschemie. 4. Aufl. Heidelberg, 1895.

PRIOR, E.: Chemie u. Physiologie des Malzes u. des Bieres. Leipzig, 1896.

ZOPF, W.: Die Pilze in morphologischer, physiologischer, biologischer und systematischer Beziehung. (Handb. der Botanik, herausg. von Schenk. Bd. iv.) Breslau, 1890.

Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten. 2 Abt. Herausg. von Oscar Uhlworm und Emil Chr. Hansen. Jena, 1895 f.

Jahresbericht über die Fortschritte in der Lehre von den Gärungsorganismen. Herausg. von Alfr. Koch. Braunschweig, 1891.

2. *Special Works on True Fungi.*

ADERHOLD, R.: Über die Morphologie deutscher Weinheferassen. (Ber. der Königl. Lehranstalt f. Obst-, Wein- u. Gartenbau zu Geisenheim a. Rh. f. 1893-94.)

——— Die Morphologie der deutschen Saccharomyces ellipsoideus-Rassen. (Landwirtsch. Jahrb., Heft 4, 1894.)

AMTHOR, C.: Studien über reine Hefen. (Zeitschrift für physiolog. Chemie. Bd. xii., Heft 1 u. 2, 1888.)

——— Über den Saccharomyces apiculatus. (Zeitschr. f. physiolog. Chemie. Bd. xii., Heft 6, 1888.)

BAINIER, G.: Observations sur les Mucorinées et sur les zygospores des Mucorinées. (Ann. d. Sc. nat. Bot., 6. Sér. Tom. xv., 1883.)

——— Nouvelles observations sur les zygospores des Mucorinées. (*Ibid.* Tom. xix., 1884.)

DE BARY, A.: Beiträge zur Morphologie und Physiologie der Pilze, III. Frankfurt, 1870.

DE BARY und WORONIN: Zur Kenntnis der Mucorineen. 1866.

Bau, A.: Der Sammelbegriff Saccharomyces cerevisiæ. (Wochenschr. f. Brauerei, 1894, No. 43.)

Behrens, J.: Die Infektionskrankheiten des Weines. (Centralbl. f. Bakt. u. Par., 2. Abt., Bd. ii., 1896, Nos. 6, 7.)

——— Beiträge zur Kenntnis der Obstfäulnis. (*Ibid.* Bd. iv., 1898.)

Beijerinck, M. W.: Die Lactase, ein neues Enzym. (Centralbl. f. Bakt. u. Par., 1889.)

——— Schizosaccharomyces octosporus, eine achtsporige Alkoholhefe. (*Ibid.*, 2. Abt., 1894.)

——— Weitere Beobachtungen über die Octosporushefe. (*Ibid.*, 1897.)

Benecke, W.: Die zur Ernährung der Schimmelpilze notwendigen Metalle. (Jahrb. f. wissensch. Botanik, Bd. xxviii., Heft 3, 1895.)

——— Die Bedeutung des Kaliums und des Magniums für Entwicklung und Wachstum des Aspergillus niger, v. Th., sowie einiger anderer Pilzformen. (Botan. Ztg., Heft 6, 1896.)

Brefeld, O.: Botanische Untersuchungen über Schimmelpilze. I., 1872; II., 1874.

Of special interest in part 1 are the researches on *Mucor Mucedo*, and in part 2, the life history of *Penicillium*.

——— Über Gärung. (Landw. Jahrb., 1874, 1875, 1876.)

Brown, A. J.: Some Experiments on the Numerical Increase of Yeast Cells. (Trans. of the Laboratory Club, Vol. iii., 1890, No. 4.)

——— The Specific Character of the Fermentative Functions of Yeast Cells. (Trans. of the Chem. Soc., 1894.)

——— Fermentative Power. An Answer to Criticism by M. E. Duclaux. (Centralbl. f. Bakt. u. Par., 2. Abt., Bd. iii., 1897, Nos. 2, 3.)

——— Influence of Oxygen on Alcoholic Fermentation. (Trans. of the Chem. Soc., 1892, Nr. 107.)

BUCHNER, E.: Fortschritte in der Chemie der Gärung. Tübingen, 1897.

Besides the above communication, Buchner has published, sometimes by himself, sometimes in conjunction with Rapp, a series of special researches in "Ber. d. deutsch. chem. Ges.," 1897, 1898 and 1899.

CALMETTE: La levûre chinoise. (Ann. de l'Inst. Past., Tom. vi., 1892.)

CASAGRANDI, O.: Über die Morphologie der Blastomyceten. (Centralblatt f. Bakt., Par. u. Inf., 2. Abt., Bd. iii., 1897, Nos. 21, 22.)

CHAMBERLAND, CH.: Recherches sur l'origine et le développement des organismes microscopiques. Paris, 1879.

CIENKOWSKI: Die Pilze der Kahmhaut. (Bull. de l'Acad. impér. des Sc. de St. Pétersbourg, Vol. xvii., 1873.)

EIJKMAN: Mikrobiologisches über die Arrakfabrikation in Batavia. (Centralbl. f. Bakt. u. Par., Bd. xvi., 1894, No. 3.)

ENGEL: Les ferments alcooliques. Paris, 1872.

ERRERA, LÉO: Sur l'existence du glycogène dans la levûre de bière. (Compt. rend. de l'Acad. des Sc., 1885.)

In connection with the above, Laurent (Annal. de l'Inst. Past., 1889) and Clautriau (Mém. publ. par l'Acad. royale de Belgique, 1895) have published later communications on glycogen in yeast. One of P. Lindner's papers mentioned below is also on this subject.

FISCHER, EMIL: Die Chemie der Kohlenhydrate und ihre Bedeutung für die Physiologie. Berlin, 1894.

——— Einfluss der Konfiguration auf die Wirkung der Enzyme II. (Ber. d. deutsch. chem. Ges., Bd. xxvii., 1894.)

Fischer, besides the above, has published several papers in conjunction with P. Lindner and H. Thierfelder in "Ber. d. deutsch. chem. Ges.," 1894 and 1895, on the enzymes contained by saccharomycetes, and on the behaviour of the latter to the different sugars.

GILTAY, E.: Pasteur und die alkoholische Gärung. (Jahrb. f. wissensch. Bot., Bd. xxx., Heft 1, 1895.)

GILTAY, E., und ABERSON, J. H.: Über den Einfluss des Sauerstoffzutrittes auf Alkohol und Kohlensäurebildung bei der

alkoholischen Gärung. (Jahrb. f. wissensch. Bot., Bd. xxvi., 1894.)

ANSEN, EMIL CHR.: Contributions à la connaissance des organismes qui peuvent se trouver dans la bière et le moût de bière et y vivre. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. i., livr. ii., 1879.)

Describes a large number of micro-organisms which belong to the various divisions of the system; gives systematic descriptions and morphological and physiological investigations. (Communications on the vinegar bacteria are to be found in the literature review of bacteria.)

—— Recherches sur les organismes qui, à différentes époques de l'année, se trouvent dans l'air, à Carlsberg et aux alentours, et qui peuvent se développer dans le moût de bière. (*Ibid.*)

—— Sur l'influence que l'introduction de l'air atmosphérique dans le moût qui fermente exerce sur la fermentation. (*Ibid.*)

—— Hypothèse de Horvath. (*Ibid.*)

—— Sur le Sacch. apiculatus et sa circulation dans la nature. (*Ibid.*, Tom. i., livr. iii., 1881.)

The first communication on this subject was published by Hansen in "Hedwigia" in 1880. The researches on the circulation of true saccharomycetes in nature are begun here and in the above paper on the organisms of the air.

—— Recherches sur les organismes qui à différentes époques de l'année se trouvent dans l'air, à Carlsberg et aux alentours, et qui peuvent se développer dans le moût de bière. 2ième Mémoire. (*Ibid.*, Tom. i., livr. iv., 1882.)

Contains not only researches on the circulation of various micro-organisms in nature, but also gives in the concluding section special investigations on several species (*Dematium pullulans* and the allied forms, *Oïdium lactis*, *Chalara mycoderma*, and various *Saccharomyces*, among them one species which gives a bitter taste to beer).

—— Les ascospores chez le genre Saccharomyces. (*Ibid.*, Tom. ii., livr. ii., 1883.)

On p. 13 are given the essentials of what was known at that time of spore formation in the saccharomycetes, also a full bibliography. On p. 23 and following, Hansen's spore cultivation method and his pure culture method for mass cultures of saccharomycetes are described. On p. 31 experiments are described, and with them are given spore curves for the six species: *Sacch. cerevisiæ I.*, *S. Pastorianus I.*, *II.* and *III.*, *S. ellipsoideus I.* and *II.* In addition, the relation of temperature to spore formation is treated in general.

HANSEN, EMIL CHR.: Sur les Torulas de M. Pasteur. (*Ibid.*)

——— Neue Untersuchungen über Alkoholgärungspilze. (Ber. der deutsch. botan. Ges., 1884.)

Researches on *Monilia candida.*

——— Vorläufige Mitteilungen über Gärungspilze. (Botan. Centralbl., Bd. xxi., 1885, No. 6.)

The gelatinous network, coalescence of spore walls, septum formation, the behaviour of *Sacch. apiculatus* under the action of sunlight.

——— Les voiles chez le genre Saccharomyces. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. ii., livr. iv., 1886.)

In addition to the investigations indicated by the title there are observations on the cell nucleus of saccharomycetes (p. 125), and new researches on the gelatinous network (p. 126).

——— Noch ein Wort über den Einfluss der Kohlensäure auf Gärung und Hefebildung. (Zeitschr. f. d. ges. Brauw., 1887, No. 13.)

——— Neue Bemerkungen zu Foth's Abhandlung: "Einfluss der Kohlensäure auf Gärung und Hefebildung". (Wochenschr. f. Brauerei, 1887.)

——— Action des ferments alcooliques sur les diverses espèces de sucre. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. ii., livr. v., 1888.)

Also descriptions of new peculiar species.

——— Untersuchungen aus der Praxis der Gärungsindustrie. (Beiträge zur Lebensgeschichte der Mikroorganismen.) 1. Heft, 1. Aufl., 1888; 3. Aufl., 1895; 2. Heft, 1892.

As regards the contents, see p. 360.

——— Über die im Schleimflusse lebender Bäume beobachteten Mikroorganismen. (Centralbl. f. Bakt. u. Par., Bd. v., 1889.)

First investigations on asporogenous varieties of *Saccharomyces* are also given here.

Hansen, Emil Chr.: Nouvelles recherches sur la circulation du Saccharomyces apiculatus dans la nature. (Ann. d. sc. nat. Bot., 7 Sér., Tom. xi., No. 3, 1890.—Ann. de Micrographie, 1890.)

Contains also researches on the circulation of true saccharomycetes.

——— Production des variétés chez les Saccharomyces. (Ann. de Micrographie, Tom. ii., 1890, No. 5.)

——— Sur la germination des spores chez les Saccharomyces. (Compt. rend. des travaux du laborat. de Carlsberg, Tom. iii., livr. i., 1891.)

——— Kritische Untersuchungen über einige von Ludwig und Brefeld beschriebene Oidium- und Hefenformen. (Botan. Zeitung, 1892, No. 19.)

——— Über die neuen Versuche, das Genus Saccharomyces zu streichen. (Centralbl. f. Bakt. u. Par., Bd. xiii., 1893, No. 1.)

——— Über künstliche und natürliche Hefereinzucht. (Zeitschr. f. d. ges. Brauw., 1895, No. 14.)

——— Experimental Studies on the Variation of Yeast Cells. (Annals of Botany, Vol. ix., 1895.)

——— Anlässlich Juhler's Mitteilung über einen saccharomycesbildenden Aspergillus. (Centralbl. für Bakt., Par. u. Inf., 2. Abt., Bd. i., 1895.)

——— Biologische Untersuchungen über Mist bewohnende Pilze. (Botan. Ztg., Heft 7, 1897.)

Besides biological researches on Coprini, the life history of *Anixiopsis stercoraria*, explanation of its life limits and contributions to the general biology of the reproductive organs.

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